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Interactions between the IGF system and cholesterol metabolism in breast cancer

Interactions between the IGF system and cholesterol metabolism in breast cancer

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Abstract

Dyslipidemia and obesity are associated with an increased risk of breast cancer. Several obesity-related host factors involved in systemic metabolism can influence tumor initiation, progression, and/or response to therapy.

Insulin like growth factor (IGF) signalling and levels of cholesterol, particularly low-density lipoprotein cholesterol (LDL-C) and its oxysterol metabolite 27-hydroxycholesterol (27OHC) are frequently up-regulated in obese women. Although high expression of LDL and 27OHC have been reported in breast cancer, their role remains to be elucidated. Triple negative breast cancer (TNBC) accounts for 50% of all deaths as they have a high affinity to metastasize to other organs. TNBC are associated with a poorer outcome because there is no specific target for therapy, such as progesterone receptor (PR), the estrogen receptor (ER)-α, or human epidermal growth factor receptor 2 (HER-2) receptor, that are expressed by other sub-types of breast cancer. The major problem for the failure of breast cancer treatment in TNBCs is the development of resistance to chemotherapy.

In this study, we examined the effect of cholesterol and its metabolite on breast cancer cell proliferation, migration and invasion and the association of the IGF axis and cholesterol metabolism. Human epithelial like estrogen receptor-α (ERα)-positive MCF-7 and T47D and ERα-negative MDA-MB-231 and Hs758T, breast cancer cells were used in this study. Crystal Violet (CV) proliferation assays were employed to detect cell growth and the

changes in cell migration were determined using a trans-well migration assay followed by crystal violet staining. Western immunoblotting was used to determine changes in protein abundance. Secreted levels of IGF-I were measured using a radioimmunoassay.

We found that LDL promotes cell growth and migration, and this action was mediated through CYP27A1 in both ERα-positive and ERα-negative breast cancer cells. Treatment with 27OHC also increased cell growth in ERα-positive breast cancer cells and this effect was mediated through ERα. 27OHC was able to promote breast cancer cell migration and invasion in all cell lines, and in the ERα-positive cells, silencing the ERα did not affect this and we found that in ERα-negative cells this action was mediated through estrogen receptor β (ERβ). The addition of LDL increased the production of IGF-I and the abundance of the IGF-IR in MCF-7 and MDA-MB-231 cells. Inhibition of the insulin-like growth factor receptor using a tyrosine kinase inhibitor, AG1024, blocked the effects of cholesterol on cell growth and migration of MCF-7 and MDA-MB-231 cells. The inhibition of insulin-like growth factor-1 receptor attenuated cholesterol-induced AKT and MAPK activation. We also found that chemotherapy treatment such as doxorubicin was less effective in inducing cell death in the presence of 27OHC or LDL in, MDA-MB-231, TNBC cells.

In conclusion, 27OHC functions as a biochemical mediator of the effects of LDL on breast cancer cell growth and migration. 27OHC promotes cell proliferation through ERα, but increased cell migration and invasion through ERβ, and this resulted in intrinsic activation of oncogenes such as IGF-1, which can bind to the IGF-1R and activate MAPK/ PI3K/AKT signalling pathways. Our data also suggest that 27OHC or LDL can act as survival factors in TNBC. The results suggest that using cholesterol lowering drugs such as, statins, may

sensitize cells to existing targeted therapies and improve the effectiveness of chemotherapy.

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programs* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Abbreviations

27OHC	27-hydroxycholesterol
BSA	Bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
dH2O	distilled water
DMSO	Dimethyl sulfoxide
DOXO	Doxorubicin
EMT	Epithelial to mesenchymal transition
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FBS	Fetal bovine serum
FUL	Fulvestrant
GM	Growth media
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
LDL	Lipoprotein, low density from human plasma
LG	L-glutamine
mTOR	Mechanistic target of rapamycin
PBS	Phosphate buffered saline
PFA/PBS	Paraformaldehyde in PBS

PHTPP	4-[2–phenyl-5,7–bis (trifluoromethyl) pyrazolo [1,5-a]-pyrimidin-3-yl] phenol
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
SDS	Sodium dodecyl sulfate
SFM	Serum free media
STAT3	signal transducer and activator of transcription 3
TAM	Tamoxifen
TE	Trypsin: EDTA

Chapter 1.

Introduction

1.1 Breast cancer

1.1.1 Breast cancer epidemiology

Breast cancer is the most common cancer worldwide in women and the leading cause of death (Bray *et al.*, 2018). The incidence rate of breast cancer currently represents one in four cancer cases and is expected to reach 3.2 million by 2050 (Bray *et al.*, 2018; Hortobagyi *et al.*, 2005). Although cancer exists worldwide, the incidence rate is greater in developed countries. Additionally, the incidence rate of breast tumour largely differs with ethnicity and race, which differs between every region in the globe differing from 27 per 100,000 in East Asia and Middle Africa to 96 per 100,000 in Western Europe (Ferlay *et al.*, 2015). However, the survival rate of breast cancer is lower in less developed countries in comparison to developed regions, and this can be explained by the lack of screening and treatment options (Torre *et al.*, 2015). Breast cancer is responsible for around 626,679 deaths globally each year (Bray *et al.*, 2018), and in the UK breast cancer is the fourth cause of cancer mortality among women ("UK Breast Cancer Statistics | Breast Cancer UK, 2020). In recent years, the American Cancer Society proposed that the incidence rate of breast cancer has increased slightly by 0.3% per year (*How Common Is Breast Cancer? | Breast Cancer Statistics*, 2019). Since 2007, breast cancer mortality rates have been steady in women less than 50 years but have continued to decline in women older than 50. Between 2013 to 2017, the death rate has decreased by 1.3% per year (*How Common Is Breast Cancer? | Breast Cancer Statistics*, 2019). Decreased mortality rates are thought to be the result of detecting breast cancer earlier through improved methods of early detection and improved targeted therapies for breast cancer (Plevritis *et al.*, 2018).

Survival rates for breast cancer significantly reduce according to stage at diagnosis, with a 5-year survival rate of 26.2% for stage 4 breast tumours, compared with 97.9% for stage one early breast cancer (CRUK, 2019). The incidence of late stage diagnosis has significantly reduced due to increased campaigns that encourage self-examination and promote awareness to increase the chance of early detection (Wang, 2017). Understanding the underlying biological mechanisms of carcinogenesis and the pathophysiology of the different subtypes of breast tumours have led to the identification of novel molecular targets and major advances in the improvement of targeted therapy (Feng *et al.*, 2018). Such targeted therapies, have enhanced both survival with metastatic disease and recurrence-free interval in the same patients including longer survival after recurrence (Perez *et al.*, 2011).

1.1.2 Normal breast development and the progression to breast cancer

The human mammary gland is an apocrine gland made of 12–20 lobes, which are made up of smaller lobules connected by a milk duct embedded in a fat pad, formed from adipocytes, immune cells and fibroblasts and infiltrated by vascular endothelial cells (Deugnier *et al.*, 2002; Macias and Hinck, 2012). The mammary epithelium consists of a basal outer layer of myoepithelial cells and a bilayer of inner luminal cells which reside on a basement membrane, and collectively these tissues are arranged as a series of branched ducts that drain alveolar structures during pregnancy, as outlined in figure 1.1A. The ducts have an outer basement membrane on which there is an outer layer of basal myoepithelial cells needed for milk ejection and a bilayer of inner luminal cells needed for milk production as outlined in figure 1.1B (Chepko and Smith, 1999). During developmental phases of

pregnancy and puberty, the mammary glands are subjected to fundamental changes (Macias and Hinck, 2012b), induced by hormonal fluctuations, notably progesterone and estrogen, that orchestrate a series of paracrine interactions between stromal and epithelial cell types to elicit phenotypic changes in the mammary gland (Sreekumar, Roarty and Rosen, 2015a).

It has been noted that the microenvironment of the breast tissue plays an important role in supporting normal breast tissue development, while restricting tumour formation; breast stroma releases regulatory signals created by extracellular matrix components. For example, laminin to enhance the formation of the normal breast architecture and cell differentiation (Silberstein, 2001).

Breast cancer is characterised by abnormal and uncontrolled growth in lobular and/or ductal epithelial cells of the breast, which can migrate to other tissues and metastasise into secondary tumours (Ingber, 2008). Cancer progression is a multistep process involving genetic alterations causing an over-proliferation of cells, which become transformed and initiate the development of solid tumours (Hanahan and Weinberg, 2011). Much work has been performed to molecularly characterize breast tumours to help understand its progression and formation (Harbeck *et al.*, 2019). Mutation and abnormal amplification of both tumour suppressor genes and oncogenes, besides the tumour microenvironment, play important roles in breast cancer progression and initiation (Bombonati and Sgroi, 2011; Hanahan and Weinberg, 2011).

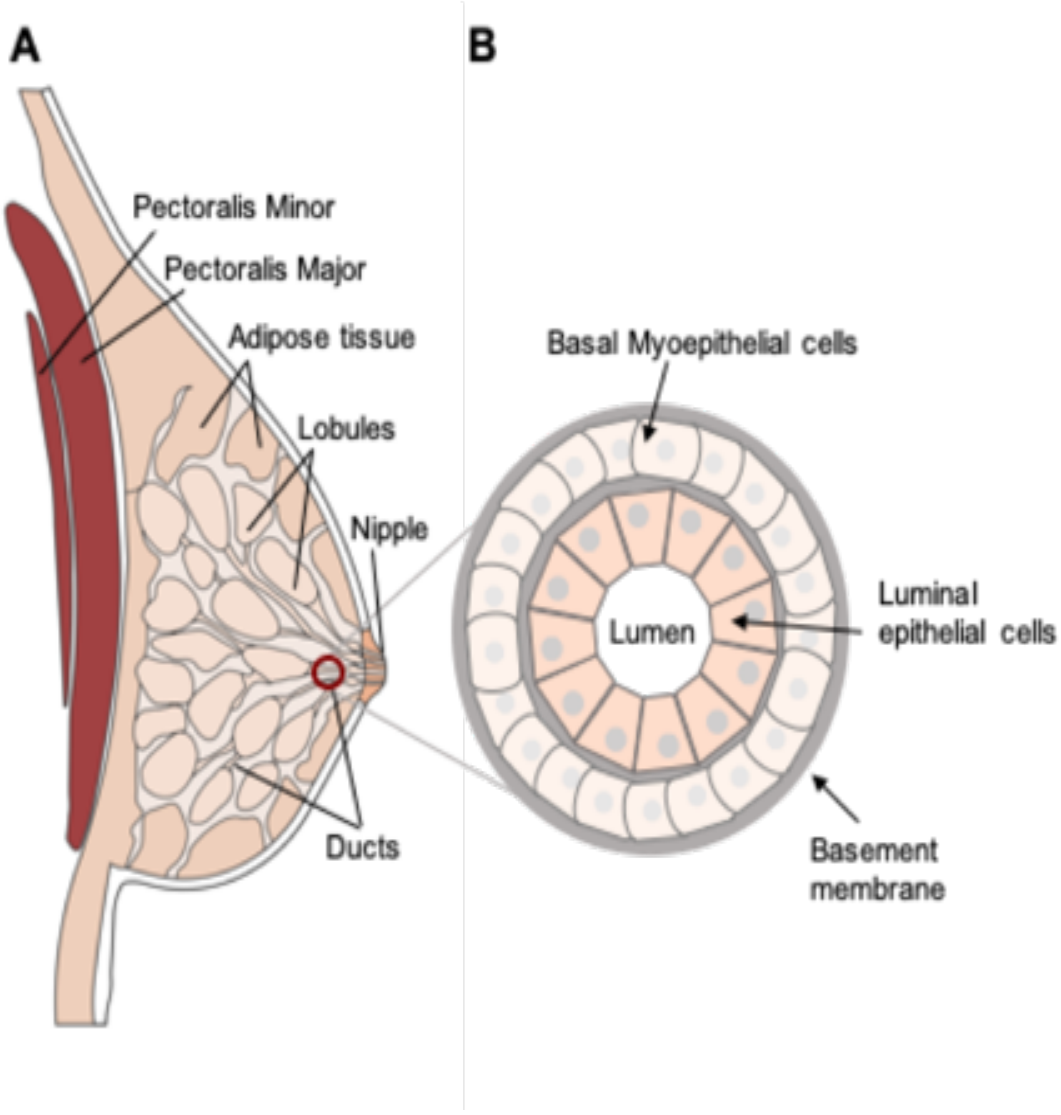


Figure 1. 1: Structure of the mammary gland.
(A) The human breast consists of lobules connected by a ductal network to the nipple, which are immersed in the stroma. Most breast cancers begin from the ducts or lobules of the breast. In more advanced breast cancer, the tumour penetrate the skin or components of the chest wall for example the pectoralis muscles (Sun et al., 2017). (B) The ducts are circled by an outer basement membrane which is bound by an outer layer of basal myoepithelial cells needed for milk ejection and a bilayer of inner luminal cells needed for milk production (Macias and Hinck, 2012), adapted from (Harbeck et al., 2019)

1.1.3 Diagnosis and screening of breast cancer

Early diagnosis of breast cancer is critical to ensure treatment starts as soon as possible. The primary aim of screening is to reduce the mortality rate and the number of women presenting with late stage cancer (Saadatmand *et al.*, 2015). A meta-analysis study of 11 randomized control trials with a 13 year follow up reported a 20% reduction in death from breast cancer among women who had been invited for screening (Bleyer and Welch, 2012). Several methods have been decided as breast cancer screening tools, such as mammography, ultrasound, magnetic resonance imaging (MRI), breast self-examination and clinical breast examination. Mammography uses low-dose Xray imaging to detect abnormalities in the breast. A secondary prevention strategy of population screening for breast cancer using mammography is proposed for diagnosing the disease at an early stage of breast cancer to allow effective treatment (Bleyer and Welch, 2012).

The UK national screening program strategy to detect breast cancer through the detection of micro calcifications and characteristic masses, is inviting women between the ages of 50-71 every 3 years for screening mammography(Marmot *et al.*, 2013). The World health organization (WHO) advise the mammography screening approach in women with age 40-49 years or 70-75 years only in the situation of careful research and in well-resourced settings (‘WHO | Breast cancer’, 2018).

Breast ultrasonography is widely available and cost-effective, and measures the acoustic waves reflected from the breast (Wang, 2017). It helps to detect cysts and solid masses, but is less efficient compared to mammography, and recommended for screening women with high density breast tissue (Hooley, Scoutt and Philpotts, 2013). In addition to using mammography, MRI enhances screening sensitivity in women with high mutation of

BRCA1/*BRCA2* and is the suggested screening application for *BRCA* mutation carriers and women with an individual history of breast cancer and those with high density breast tissue (Saslow *et al.*, 2007). The treatment decisions are taken after evaluating the grade/stage of the cancer and biological characteristics, menopausal status, the patient's age and preferences and the risks and benefits associated with the particular option. However, the early management plan in breast cancer is based on loco-regional tumour load, molecular subtype, and patient decision (Harbeck and Gnant, 2017).

1.1.4 Breast cancer grading and staging

Cancer cells are given a grade and stage, which is used to predict prognosis. Histological grading depends on the microscopic appearance of cancer cells, which measures the percentage of the degree of nuclear pleomorphism, tubule formation and numbers of mitotic figures (Harbeck and Gnant, 2017). A score of 1 to 3 is provided based on the degree of abnormality of the breast tumour cells for each criteria; the scores are then added and assigned a grade (from 1 to 3) (table 1.1) (Elston and Ellis, 1991).

Table 1. 1: Breast cancer grading, adapted from (Elston and Ellis, 1991; Bloom and Richardson, 1957).

Feature graded	Clinical feature	Score
Tubule formation	> 75%	1
	10-75%	2
	< 10%	3
Nuclear polymorphism	Small, regular uniform cells	1
	Moderate increase and variability	2
	Marked variation	3
Mitotic figures (per 10 higher power fields)	0- 5	1
	6- 10	2
	>11	3

Grade	Total score
1	3-5
2	6-7
3	8-9

Breast cancer is graded based on histological features; then combined the score for each of these and add this score to the final grade as described.

From 1959, seven editions of the tumor-node-metastasis (TNM) system have been established for tumour staging from the American Joint Committee on Cancer (AJCC). The 8th edition was published in 2017, as outlined in table 1.2. The fundamental changes focus on breast cancer as a group of diseases including multiple molecular characteristics that demonstrate patterns of recurrence, various prognoses, and sensitivities to available therapies (Elston and Ellis, 1991). The committee integrated biomarkers (hormone receptor, histologic grade, multigene panels, and *HER2* expression) into the traditional

anatomic TNM staging. Breast cancer is then categorised into four stages 1-4 dependent on the TNM criteria (Koh and Kim, 2019). Following staging and grading of breast tumours, histological examination of the excised tumour is required for evaluation of the subtype of breast cancer to decide the appropriate treatment (Koh and Kim, 2019). Breast cancer is staged depending on the guidelines of the American Joint Committee on Cancer (AJCC), following the TNM evaluation of primary cancer size (T), degree of lymph node involvement (N) and extent of metastasis (M) (Ravaioli and Tassinari, 2000) as outlined in table 1.2.

Table 1. 2: Staging criteria for breast cancer tumours: adapted from (Egner, 2010; Koh and Kim, 2019).

TNM Class	Clinical Features	Stage Group	
Tumour(T)		Stage 1	T1 N0 M0 T0 N1 M0
Tx	Primary tumour cannot be measured	Stage 2A	T1 N0 M0 T2 N1 M0
T0	Primary tumour cannot be found	Stage 2B	T2 N0 M0 T3 N1 M0
T1	Tumour <2 cm	Stage 3A	T0 N2 M0 T1 N2 M0 T2 N2 M0 T3 N1-2 M0
T2	Tumour > 2 cm but < 5 cm nodes or <5cm in size and	Stage 3B	T4 N0 M0 T4 N1 M0 T4 N2 M0
T3	Tumour > 5.0 cm	Stage 3C	Any T N3 M0
T4	Tumour of any size with direct extension to the chest wall or skin	Stage 4	Any T Any N M1
Lymph Node (N)			
NX	Cancer in nearby lymph node cannot be measured		
N0	No regional lymph node metastasis		
N1	Metastasis in movable ipsilateral axillary lymph node (s)		
N2	Metastasis to ipsilateral axillary lymph node (S) fixed or matted.		
N3	Metastasis in ipsilateral infraclavicular lymph nodes with or without axillary lymph node involvement		
Metastasis(M)			
MX	Cancer in nearby lymph node cannot be measured		

Breast cancer is staged based on the amount of invasion/migration to lymph nodes (N), tumour size (T), and surrounding tissues (M). According to TNM classification, and these scores are categorised into 1-4 stages.

1.2 Breast cancer subtypes

The two major approaches in the management of breast tumour are systemic therapy and locoregional treatment; the molecular alterations and histological characteristics of breast cancer dictate breast cancer management and treatment (Sims *et al*., 2007). There are several molecular pathways that are involved in the development of breast carcinogenesis and establish various categorisations. The intrinsic classification, reported in 2000 by Perou, categorised subtypes of breast cancer into luminal-A and -B (having high expression of the estrogen receptor (ER), basal-like and human epidermal growth factor receptor 2 (HER2)-positive (without ER expression) (Perou *et al*., 2000; Sørlie *et al*., 2001). This classification shifted clinical diagnosis from being dependent on tumour burden to being dependent on the biology of the tumour. Nowadays, clinical practice classifies tumours based on five subtypes, that encompass the histological and molecular characteristics. Tumours expressing a progesterone receptor (PR) and/or estrogen receptor or/and HER2 are classified as hormone receptor-positive breast cancers: two ER-positive (ER-positive; luminal A and luminal B), two ER-negative (ER−; ERBB2 and basal subtypes), and triple-negative breast cancer (TNBC: that do not express PR, ER or HER2) (Perou *et al.,* 2000). Women with the ER-positive or PR-positive /HER2-positive subtype have better survival outcomes than those with the ER-negative or PR-negative /HER2-negative subtypes (Howlader *et al*., 2018).

The best survival rate was between women with the ER+ or PR+/HER2+ subtype, whilst the shortest survival rate was between women with ER- or PR-/HER2- breast tumor subtype (Li *et al*., 2020).

The inadequate approach to molecular genetic tests in addition to their high costs limits its use in clinical practice, therefore, other more feasible approaches such as immunohistochemical (IHC) markers are used to predict breast cancer subtypes (Reis-Filho and Pusztai, 2011). Tumour growth marker is a nuclear marker, Ki-67, that is expressed in all phases of the cell cycle, except G0. Ki-67 is a crucial determinant of breast tumour prognosis and staging system, as an algorithm is used to decide the most appropriate medication for patients with breast cancer. Ki67 is only applicable for ER+, HER2- breast tumours, since HER2+ breast tumours and TNBC subtypes require chemotherapy treatment, and assessment of Ki67 does not contribute to medical treatment decision-making comparing to other breast cancer subtype.

The tumour grade is determined by evaluating morphological characteristics and by assessing Ki-67 expression. A cut-off point of 20% was determined to differentiate high Ki-67 from low Ki-67 to discriminate between luminal A-like and luminal B-like breast tumours and thereby the need for added chemotherapy, in the St. Gallen Consensus (Gnant, Harbeck and Thomssen, 2011). The low growth tumors were defined at Ki-67 value <20%, cut-off and the high growth tumours were defined at ≥20% Ki-67 value (Cheang *et al*., 2009).

Luminal A-like was categorised as ER+/HER2- with low Ki67 and PR ≥ 20%. Therefore, luminal B (HER2-) was categorised as ER+/HER2− with high Ki67 or PR < 20%. Ki67 cut-off value 20% stained nuclei is the most widely used in invasive cancer cells and is considered low growth rate at a cut-off of <15% stained nuclei (Nielsen *et al*., 2020).

Assessing Ki-67 is important in determining the best adjuvant chemotherapy treatment for patients with hormone receptor-positive breast tumours.

1.2.1 Luminal breast cancer

Luminal A breast cancers are characterised as a low risk group that reveal a good response to endocrine treatment, compared to luminal B subtype, which has a higher grade and higher expression of Ki-67 (Perou *et al.*, 2000). New definitions and guidelines for the management of luminal-A and -B were advised in St Gallen consensus 2011 (Goldhirsch *et al.*, 2011). The cutoff point between high and low expression of Ki-67, which is the value of Ki-67 of 14% was the one best associated with the gene expression that related to luminal A type, whereas the high value for Ki-67 $\geq$14 was used as new definition for the HER2-negative luminal-B subtype. In the ST. Gallen, 2013, consensus meeting, another new surrogate definition, reduced PR expression, was added for the HER2-negative luminal-B subtype, with a PR expression cutoff point of $\geq$20% related to luminal A subtype(Goldhirsch *et al.*, 2013), as described in table 1.3 (Perou *et al.*, 2000) such as, ER- and PR- related genes, lack of gene expression of HER2, and low expression of proliferation-related genes (Bombonati and Sgroi, 2011). Luminal A are the most frequent type of breast cancer, with a frequency 28-31% (Sotiriou *et al.*, 2003).

Luminal B breast cancers are defined by ER positivity, higher expression of Ki-67 and the HER2 gene and a lower expression of different luminal-related genes, such as estrogen receptor 1 gene (ESR1) or Forkhead Box A1 (FOXA1) (Prat *et al.*, 2015), compared to luminal-A type.

Subtype	**Characteristic**	**Treatment**
Luminal A	ER(+) &PR(+) & HER2(-)	Hormonal therapy
Luminal B	ER(+) & PR(+) & HER2 (-/or +)	Hormonal and /or chemotherapy

Table 1. 3: Breast cancer molecular sub-types and treatment

Luminal B tumours are linked with a poorer prognosis and are likely to have a greater risk of a recurrent cancer than that of luminal A (Perou *et al.*, 2000). The two luminal A and B are categorised with positive gene expression of the hormone receptors, ER and PR, although a portion of luminal B subtype also express HER2, as described in table 1.3 (Ahn *et al.*, 2015). Furthermore, the two ER-positive breast cancer subtypes, are correlated with long term survival and a good prognosis, in comparison to ER-negative subtypes (HER2-positive and basal-like) that are linked with poor prognosis, and this difference is due to an individual response to antiestrogen treatment (Cheang *et al.*, 2009).

1.2.1.1 Estrogen receptors:

Estrogen plays a crucial role in the development of the normal reproductive system: being involved in regulation of the immune system, bone, brain, reproduction, cardiovascular system, breast, prostate, and inflammatory processes (Minutolo *et al.*, 2011). Estrogen is also a promotor of breast cancer, through its binding to the ER (encoded by estrogen receptor 1(ESR1)), which dimerises and translocates to the nucleus, where it binds to ER elements (EREs), to regulate the expression of various genes (Osborne *et al.*, 2001), such as those linked with cell proliferation, survival and growth including cyclin D1, insulin-like growth factor-1 receptor (IGF-1R) (Yu *et al.*, 2012), anti-apoptotic B-cell lymphoma-2 (BCL-2) protein and vascular endothelial growth factor (VEGF) (Fotovati *et al.*, 2006; Kousidou *et al.*, 2008). Additionally, the ER also regulates non-genomic effects, by binding to cell surface receptors and activating cell signalling pathways. G-protein coupled estrogen receptor 1 (GPER) has been proposed to initiate non-genomic actions of estrogens in the cytosol. This leads to activation of several signalling pathways; phosphatidylinositol-3-OH kinase (PI3K)/protein kinase B AKT (Lee *et al.*, 2005), protein kinase C (PKC),

mitogen activated protein kinases (MAPKs)(Kleuser *et al.*, 2008), to modulate various functions within the cell, such as, cell proliferation and survival (Lipovka and Konhilas, 2016), explained in figure 1.2. During normal breast development, the hormones progesterone and estrogen are mainly responsible for coordinating the changes that occur in females through the activation of different transcription factors responsible for cell growth and differentiation (Sreekumar, Roarty and Rosen, 2015b). However, during the menstrual cycle the imbalance between progesterone and estrogen enhances the constitutive activation of the estrogen signalling cascades along with acquired mutations of oncogenes, that may promote the uncontrolled or abnormal growth of mammary epithelial cells and enhance the development of cancer cells (Feitelson *et al*., 2015). Furthermore, there is a strong correlation between postmenopausal women and the development of ER-positive breast cancer, that is thought to be due to the effects of high estrogen levels in obese women which leads to increased breast cancer progression and growth (Omoto *et al.*, 2002; Murillo-Ortiz *et al.*, 2008; Cheng *et al.*, 2013). In post-menopause women, the main source of estrogen modulation is the adipose tissue. Therefore, dysregulation of ER signalling is linked with progression and initiation of several cancers, including breast cancer.

The action of estrogen is predominantly regulated via its two receptors: ERβ and ERα, which have different transcriptional activities in normal breast development and breast tumour progression (Osborne *et al.*, 2001). The effects of estrogen are mediated through activation of various genes that are regulated by ligand-modulated transcription factors, up- and/or downregulating gene expression in target tissues(Minutolo *et al.*, 2011).

ERα is expressed in 50–80% of breast cancers and is a significant indicator for breast tumour survival and the efficacy of hormone therapy. ERα is expressed less in normal human mammary epithelial cells of postmenopausal women, approximately by 10%, whilst ERβ expression level is higher; approximately by 50% in normal mammary epithelial cells of postmenopausal women, but not for premenopausal women. Nuclear ERβ but not ERα expression is found in stromal cells in postmenopausal women (Omoto *et al.*, 2002; Murillo-Ortiz *et al.*, 2008; Cheng *et al.*, 2013). In breast cancer patients, loss of ERα indicates invasiveness and poor prognosis (Cheng *et al.*, 2013).

The primary function of ERβ in breast cancer progression is still being investigated (Lipovka and Konhilas, 2016). ERβ is expressed more than the ERα in mouse mammary glands and normal mammary epithelium (Roger *et al.*, 2001). ERβ is also found in both myoepithelial cells and surrounding endothelial and stromal cells of the breast. It is purported that the role it plays depends on the relative ratio of expression of ERα to ERβ, and recent data suggests that ERβ has a pro-tumourigenic role in ERα-negative breast cancers (Leygue and Murphy, 2013). Several studies have reported on the expression of ERβ in patients samples, and some of these studies have found a link between high ERβ expression level and good prognosis in breast cancer(Järvinen *et al.*, 2000; Hopp *et al.*, 2004; Esslimani-Sahla *et al.*, 2005; Miller *et al.*, 2006; Gruvberger-Saal *et al.*, 2007; Sugiura *et al.*, 2007a; Bozkurt and Kapucuoĝlu, 2012). Additionally, current pre-clinical studies in breast tumour animal models and cell lines propose a beneficial impact of ERβ (Warner and Gustafsson, 2010; Murphy and Leygue, 2012).

The importance of ERβ in breast cancer progression and development is still ambiguous, as ERβ plays various functions based on the presence or absence of ERα (Haldosén, Zhao and Dahlman-Wright, 2014a). In fact, approximately 60% of all subtypes of breast tumours, that tested positively for high ERβ expression, do not have ERα expression (Skliris *et al.*, 2006a). Previous studies proposed that ERβ is thought to act as a carcinogenic factor in breast tumours (Haldosén, Zhao and Dahlman-Wright, 2014b). Notably, in recent studies ERβ is defined as a predominant negative regulator of estrogen pathway, hence ERβ restrained ERα regulated transcription through creating heterodimers with ERα (Girgert, Emons and Gründker, 2019). The nuclear ERβ is expressed in 44.4% of TNBCs (Matthews and Gustafsson, 2003; Marotti *et al.*, 2010). ERβ has five different isoforms, therefore these multiple isoforms complicate the investigation of the function of ERβ and its participation in breast cancer carcinogenesis (Girgert, Emons and Gründker, 2019). The role of ERβ in TNBC is not yet clear. A study analyzed 442 tissue samples from women with breast cancer administered with tamoxifen for the effect of ERβ1 expression on overall survival rates (Honma *et al.*, 2008). Those groups with ERβ1-positive tumors demonstrated a significantly better overall survival rate in comparison to women with ERβ1-negative group in postmenopausal women, but not premenopausal women (Honma *et al.*, 2008). Patients with progesterone-receptor negative, ERα-negative breast cancer expressing ERβ1 displayed a better prognosis regardless of whether the tumors with HER2-positive or not (Zhang *et al.*, 2012).

The remaining four isoforms of ERβ (ERβ2, ERβ3, ERβ4, and ERβ5), do not bind to estrogen (Honma *et al.*, 2008; Zhang *et al.*, 2012). Several reports explored the influence of nuclear ERβ-isoforms expression on the prognosis of breast cancer. It has

been proposed that a better overall survival rate for breast cancer patients is evident with a high expression of both ERβ1 and ERβ2, and that ERβ1 expression is strongly associated with ERβ2 expression (Sugiura *et al.*, 2007b). Interestingly, it has been proposed that nuclear ERβ1 and ERβ5 correlated with better overall survival rates in a study of 880 breast cancer patients (Shaaban *et al.*, 2008).

In contrast, Novelli and their collogue indicated that ERβ expression is a biomarker related to more aggressive proliferation in lymph node-positive breast cancer patients and indicated a higher chance of relapse (Novelli *et al.*, 2008a). Additionally, ERβ inhibits estrogen-induced proliferation by preventing ERα activation of the cyclin D1 gene, a major modulator of entry into the proliferative step of the cell cycle, when both receptors were expressed in Hela cells. This suggests that ERβ is capable of regulating the growth effects of 17β-estradiol binding to ERα (Liu *et al.*, 2002). A recent study has been reported that ERβ2 is associated with growth and invasion of TNBC through the regulation of HIF-1 pathway (Bialesova *et al.*, 2017).

In TNBC, high expression of the epidermal growth factor (EGF) receptor is considered as one hallmark of high proliferation rates in these cancer types (Chen and Russo, 2009). Studies have reported a correlation between ERβ and the insulin-like growth factor (IGF) and EGF signalling pathways (Thomas *et al.*, 2012). Knocking down ERβ in TNBC cell lines reduced cell proliferation, invasion and migration that were linked with down-regulation of the IGF-I and EGF receptors (Richardson *et al.*, 2011; Hamilton *et al.*, 2015).

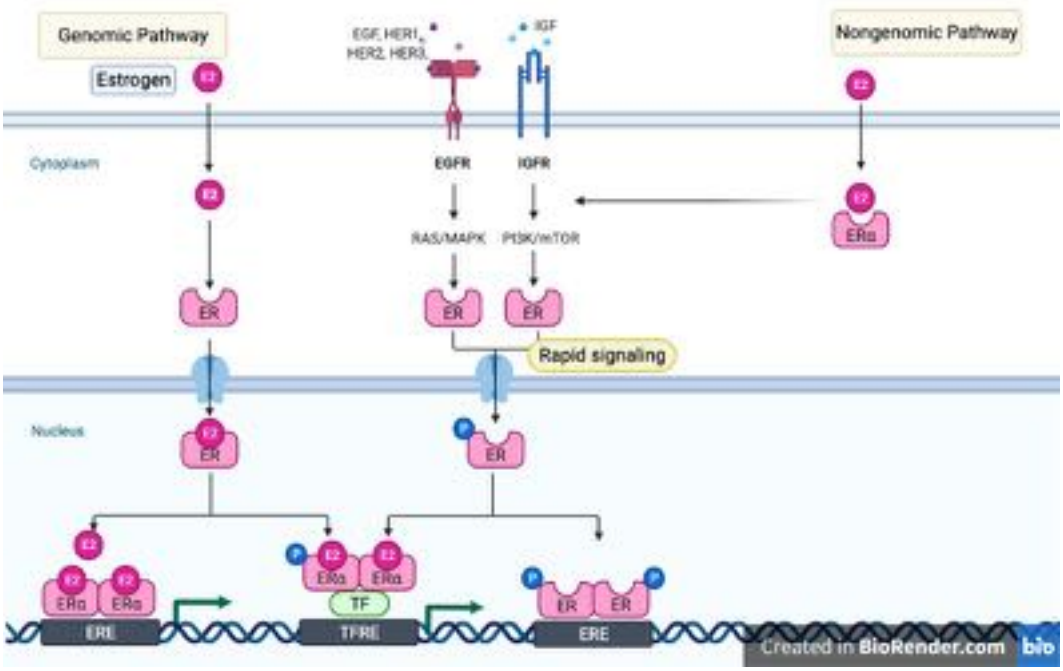


Figure 1. 2: A diagram of estrogen signaling pathways, including genomic and non-genomic response structures, adopted from (Tsuji and Plock, 2017)**.**
The arrow indicates the direction of the reaction. (ER) estrogen receptor, (ERE) estrogen responsive elements, (E) estrogen, HER, human epidermal growth factor receptor; (MAPK) mitogen-activated protein kinase, SRC proto-oncogene, non-receptor tyrosine kinase, P, protein; PI3K phosphatidylinositide 3-kinase, AKT protein kinase B; mTOR, mammalian target of rapamycin;(TF) transcription factor.

1.2.1.2 Targeted therapy for ERα-positive breast cancer

Targeted therapy for ER-positive breast cancer includes tamoxifen, an ER antagonist, that prevents estrogen-induced effects by inhibiting estrogen receptors in breast tissues (Piperigkou *et al.*, 2016). Aromatase inhibitors (AIs) have been used as targeted therapy for ERα-positive breast cancer, which prevent the conversion of androgens, such as testosterone, to estrogen, and result in a reduction of estrogen production and ER activation (Miki *et al.*, 2007). Intra-tumoural production of estrogen can be increased by cancer cells and this is catalyzed by the cytochrome P450 aromatase enzyme, a major enzyme in

estrogen synthesis and present in breast adipose stroma and epithelium, which activates estrogenic signalling pathways (Brown and Simpson, 2010). Preclinical studies have advocated that aromatase inhibitors are likely to be more effective than tamoxifen in the treatment of ER-positive breast tumours in postmenopausal women, due to the serious and long-term side effects of tamoxifen in the treatment of advanced breast tumours (Xu, Liu and Li, 2011; C. Y. Liu *et al.*, 2017). Significantly, these endocrine targeted therapies for ER-positive breast cancer have decreased mortality rates, by approximately 25-30% (Haque and Desai, 2019). However, about 30% of women treated with endocrine targeted therapy, in particular tamoxifen, develop the risk of recurrence, resistance in the next decade and long-term toxicities such as thromboembolic events and endometrial cancer, and this causes increasing use of alternative hormonal therapies such as aromatase inhibitors (Dowsett *et al.*, 2010; Xu, Liu and Li, 2011). However, not all ER-positive breast cancer patients respond to targeted therapy for ER-positive breast tumours, and even those that are initially responsive ultimately become resistant as the disease progresses (Haque and Desai, 2019).

Emerging evidence suggests several pathways can contribute to endocrine therapy resistance by ligand-independent activation of ER, such as growth factor activated pathways, PI3Kinase/AKT and MAP kinase, and G-protein coupled receptor pathways (Haque and Desai, 2019). Furthermore, ER-positive breast cancers which overexpress growth factors like HER2 and EGFR are more likely to become resistant to hormone therapy (Haque and Desai, 2019). Clinical trials suggested that using PI3K/AKT inhibitors combined with aromatase inhibitors resulted in promising targeted therapy for ER-positive /HER2-positive tumours with PI3K/AKT hyper-activation (Miki *et al.*, 2007). Therefore,

understanding the involvement of metabolic proteins in growth factor signalling, in addition to ER, is a developing strategy to stop the development of resistance to endocrine therapy.

Preclinical models using a CDK4/6 inhibitor, Palbociclib, in hormone receptor positive breast cancer studies indicated high sensitivity and a good response rate. The cyclin-dependent kinases 4 and 6 (CDK4/6) are major promoters of cancer proliferation that regulate cell cycle progression in hormone receptor-positive breast tumours, correlating with estrogen receptor signalling pathway stimulation (Turner *et al.*, 2018). Palbociclib when added to the endocrine therapy letrozole (O'Leary *et al.*, 2018) and fulvestrant for cure of hormone receptor positive and HER2-negative metastatic breast tumour increased progression free survival (Cristofanilli *et al.*, 2016).

1.2.1.3 HER2-positive breast cancer:

HER2-positive breast cancer is defined by loss of expression of PR and/or ER gene, HER2 amplification and the low expression of basal and luminal clusters as described in table 4 (Lee, Loh and Yap, 2015). HER2 (named ERBB2) is one member of the epidermal growth factor (EGFR) family of receptor tyrosine kinases (RTK) along with HER1, HER3 and HER4 (Perou *et al.*, 2000; Kondov *et al.*, 2018). HER2 signalling is a crucial mediator of cell differentiation and proliferation during normal breast development. However, amplification of *HER2,* leads to increased expression of the receptor which is linked with the development of HER2-positive breast cancer (Yarden and Pines, 2012) through the activation of downstream signalling cascades, such as the PI3K and MAPK signalling cascades (Ono and Kuwano, 2006; Burgess, 2008). Between 15-20% of all breast cancer

cases are diagnosed with HER2-positive breast tumours and are largely correlated with more aggressive breast diseases, shorter disease-free survival and higher recurrence rates (Cho *et al.*, 2003; Iqbal and Iqbal, 2014).

1.2.1.4 Targeted therapy for HER2 breast cancer

Trastuzumab (Herceptin) is a targeted therapy, that is used to target HER2 genes for HER2-positive breast cancer, which prevents the dimerization and activation of HER2, as described in table 4 (Vu and Claret, 2012). Trastuzumab results in the inhibition of the downstream PI3K and MAPK signalling cascades, which leads to the suppression of cell growth and proliferation (Vu and Claret, 2012). Pertuzamab is a targeted therapy, which inhibits the dimerization of HER3 and HER2 receptors and is also used for HER2-positive breast tumours in combination with trastuzumab to inhibit the HER2 signalling cascade (Vu and Claret, 2012), which has significant results with HER2-positive metastatic breast cancer (Capelan *et al.*, 2013). A clinical study found that IGF-1R expression increased with trastuzumab resistance (Kast *et al.*, 2017). Hence, understanding the molecular mechanisms underlying resistance to therapy may reveal novel targets to improve therapeutic benefit.

Table 1. 4: Breast cancer molecular sub-types and treatment

Subtype	Characteristic	Treatment
HER2-enriched	ER- and /or PR-, HER 2+	Targeted immunotherapy, (Trastuzumab)

1.2.2 Triple-negative breast cancer (TNBC)

The TNBC subtype is defined as ER-negative, HER2-negative, PR-negative with high expression of Ki-67, as described in table 1.5 (Vu and Claret, 2012; Harbeck and Gnant, 2017). TNBC accounts for approximately 20% of all diagnosed breast cancers, but is responsible for roughly 50% of all breast cancer mortalities as they have a high propensity to metastasize to the critical visceral organs (Chavez, Garimella and Lipkowitz, 2010). Patients with TNBC have a poorer outcome in comparison with the other diagnosed sub-types of breast cancer principally due to no specific targetted therapy for TNBC (Chavez, Garimella and Lipkowitz, 2010).

TNBC is more common in women less than 40 years of age, in women with *BRCA1* gene mutations and among African-American women (Gonzalez-Angulo *et al.*, 2011; Allison, 2012). In TNBC, high expression of epidermal growth factor (EGF) is one hallmark for increasing proliferation of these tumors. TNBC at diagnosis is more likely to be T2 or T3, and have already metastasised to the lymph nodes and are more aggressive than other breast cancer subtypes (Girgert, Emons and Gründker, 2019). TNBC are difficult to detect by screening methods like mammography because of the higher breast tissue density or rapid growth rate leading to late detection (Dent *et al.*, 2007).

The lack of a specific target is the major issue for the development of specific treatments against TNBC (Dent *et al.*, 2007). Approximately 70% of TNBC subtypes are classified as basal-like tumours because both are heterogeneous subgroups which illustrate the poorest prognosis of this subtype (Lachapelle and Foulkes, 2011). Basal-like tumours are more likely to be characterised by the expression of individual genes of the epithelial cells in the outer layer or basal of the mammary gland for example EGFR and cytokeratin 5 and 17

(Shah *et al.*, 2012). Upregulation of basal marker genes such as cytokeratin 5 and 17 result in deregulation of genes involved in the cell cycle pathway, alteration of growth factor signalling and DNA damage response pathways leading to a promotion of cell proliferation and tumour progression (Lehmann *et al.*, 2011).

Table 1. 5: Breast cancer molecular sub-types and treatment

Subtype	Characteristic	Treatment
Triple-negative/basal- like	ER-, PR- and HER2-	Chemotherapy

1.2.2.1 TNBC management

Since TNBCs lack druggable targets, such as HER2 and ER, chemotherapy is still the main choice of treatment for this sub-type of breast cancer (Lebert *et al.*, 2018). Neoadjuvant chemotherapy results in higher rates of pathological complete response for localised early-stage TNBC compared with hormone-positive breast cancers (28% vs. 6.7%) (Nakai, Hung and Yamaguchi, 2016). In particular, patients who have defective DNA repair mechanisms for example those with high expression of *BRCA1*/*BRCA2* mutations, respond positively to chemotherapy (Lebert *et al.*, 2018). Although, TNBC indicates a favourable clinical response to chemotherapy, overall and cancer specific survival rates are lower than the other breast tumour subtypes, which is largely attributed to their more aggressive phenotype (Wahba and El-Hadaad, 2015). Although, it has been demonstrated that the EGFR signalling pathway plays an important role in TNBC progression, the results from clinical studies in breast cancer of EGFR-targeted therapy have been disappointing due to low response rates (Masuda *et al.*, 2012). Another factor to consider is resistance to EGFR-targeted therapies, promoted by the upregulation of

down-stream signalling-independent of EGFR activation (Nakai, Hung and Yamaguchi, 2016).

Furthermore, the most promising clinical target for TNBC is poly (ADP-ribose) polymerase (PARP). Inhibitors of PARP induce synthetic lethal actions in breast cancer patients with high *BRCA* mutations and interfere with the repair of single-stranded DNA breaks leading to tumour cell apoptosis (Nakai, Hung and Yamaguchi, 2016).

1.3 The insulin-like growth factor system

The insulin-like growth factor (IGF) system is a complicated network which plays an important role in the regulation of cell growth, and survival. It is essential in embryonic and postnatal development, as it influences almost all organ systems. IGFs were discovered in the early 1950s as somatomedins, however, when their insulin-like actions were identified later, the term insulin-like growth factors was then introduced in 1976 (Steck and Murphy, 2019)

The IGF ligands, IGF-I and IGF-II, have high structural similarity to insulin.

The physiological responses to specific receptor activation is still under investigation. In general, binding to the IGF-1 receptor (IGF-IR), is thought to mediate mitogenic effects, with the insulin receptor (IR) mediating metabolic actions. Each ligand has higher affinities for their corresponding receptors but can also bind to other receptors with lower affinity.

IGF-II is able to bind to the IGF-IR receptor (6 fold lower affinity than IGF-I) and to the IR with different affinity. The IGF-IR is thought to mediate most of their biological functions (Werner, Weinstein and Bentov, 2008).

The IGF system also has six high affinity IGF binding proteins (IGFBP-1 to 6) that can have effects that are dependent or independent of IGF binding (Rinderknecht and Humbel, 1976; Baxter, 2014).

1.3.1 Insulin like growth factors–I (IGF-I &-II)

IGF-I has 50-70% similarity to the structure of insulin. This enables IGF-I to bind (with low affinity) to the IR (Cox *et al.*, 2015). IGF-I is primarily secreted by the liver into the blood circulation (Rechler, 1993). IGF-I is also expressed in most tissues including breast tissue. IGFs consist of the insulin growth factors (IGF-I and IGF-II), and their cell membrane receptors (IGF-IR, IGF-IIR, and IR) and six high-affinity binding proteins (IGFBP-1 to -6) which act primarily as carriers for IGFs. Most tissues can make IGFs and IGFBPs.

The predominant regulator of IGF-I is growth hormone (GH) and it generally is involved in cell proliferation, migration and apoptosis, therefore affecting organisms longevity and size (Rechler, 1993).

Although IGF-I is mainly considered a key factor in postnatal growth, which stimulates the growth of several cell types, it has other significant metabolic effects, such as direct effects on specific tissue or not direct effects by mediating the actions of GH. Additionally, IGF-I reduces blood glucose levels by enhancing tissue glucose uptake in muscle and blocking glucose production from the liver and it helps to increase insulin sensitivity (Zapf, Schmid and Froesch, 1984) and to promote protein synthesis. IGF-I serves as a mediator of mammary branching and ductal formation, which is implicated in the pathogenesis of breast tumours (Belardi *et al.*, 2013). Importantly, IGF-I is reported as having a significant

effect in cancer progression, including breast cancer and in addition in contributing to resistance to targeted therapies (LeRoith and Roberts, 2003; Christopoulos, Corthay and Koutsilieris, 2018a).

IGF-II is a 7.5 kDa peptide primarily secreted by the liver, with 62% homology with IGF-I and consists of 67-amino acids. Serum levels of IGF-II are stable in adults, with a mean concentration of 700 ng/ml and a minimal decline with ageing (Humbel, 1990). IGF-II serum levels are correlated to the nutritional habits of the individual, as its levels have been shown to decrease with malnutrition (Wolf *et al.*, 1994) and increase in overweight individuals, (Buchanan, Phillips and Cooper, 2001) both total and free IGF-II (Frystyk *et al.*, 1999); this alteration in serum levels being reversible with weight loss (Belobrajdic *et al.*, 2010). The physiological role of IGF-II is less well understood during adult life, but in comparison with IGF-I, IGF-II is more abundant in the blood serum and remains stable during pregnancy as a regulator of fetal development and growth (Frasca *et al.*, 1999). The lack of clear understanding of the role of IGF-II may be due to its overlapping effects with IGF-I (Frasca *et al.*, 1999). IGF-II is an important factor for normal embryonic growth but not important for postnatal development in mice (DeChiara, Efstratiadis and Robertsen, 1990). Several studies have shown that IGF-II plays a role in intra-uterine proliferation in humans, as it is plentifully secreted by the placenta; IGF-II also helps to promote the formation of mesoderm cells, trophoblast invasion and growth, survival of cytotrophoblasts, enhance organ development and improve nutrient transport. IGF-II promotes angiogenesis through its regulation of vascular endothelial growth factor (VEGF), which can stimulate differentiation of endothelial cells (Irwin *et al.*, 1993; Schwartz, Hudgins and Perdue, 1993). A recent study suggested that IGF-II is also crucial

in growth promotion in humans after birth (Begemann *et al.*, 2015) and in postnatal bone development and cartilage proliferation (Uchimura *et al.*, 2017).

1.3.2 IGF-I receptors

The mitogenic actions of IGF-I are modulated primarily via the IGF-IR receptor (Ward *et al.*, 2013). IGF-I receptor activation affects cell proliferation, migration, survival and invasion (Arcaro, 2013). The IGF-IR and IR belong to the tyrosine kinase family of receptors and they share almost 84% structure similarity. These two cell membrane receptors consist of α and β subunits connected by disulphide bonds to form homodimers (Laron, 2001). The α subunit is the ligand binding site and located extracellularly, while the β subunits are transmembrane and contain tyrosine kinase domains (Werner, Weinstein and Bentov, 2008), as described in Figure 1.3.

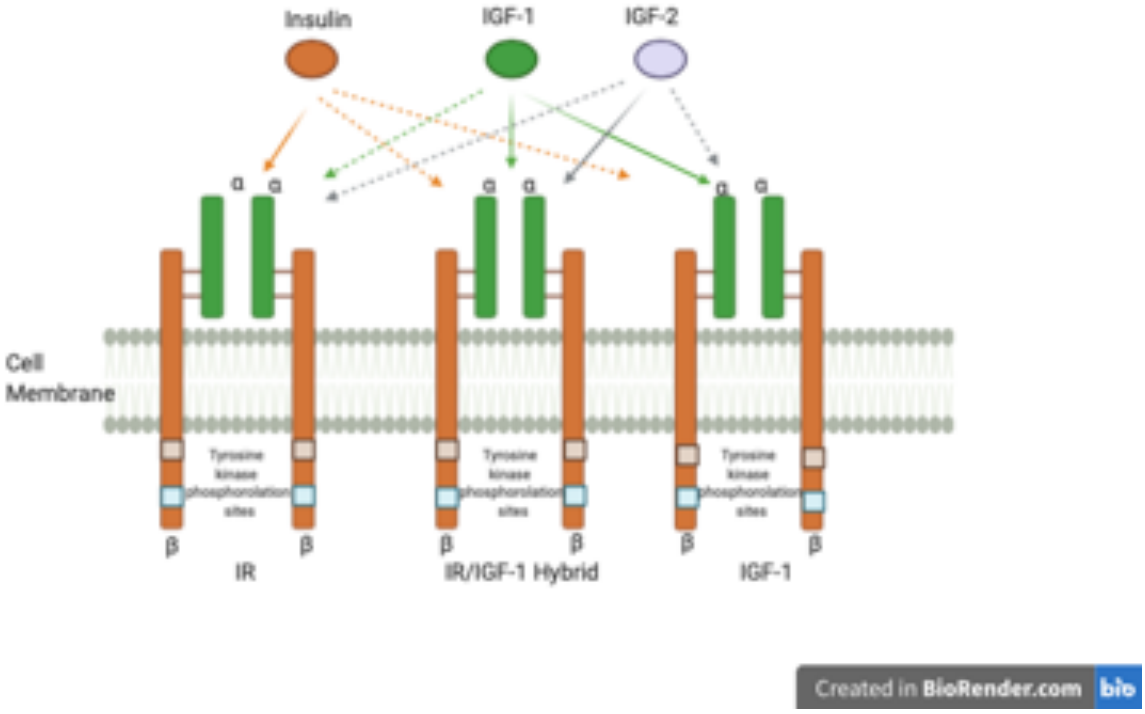


Figure 1. 3: Structure of the IGF receptors
The insulin structure, IGF-I structure, insulin/IGF-I hybrid receptors structure. IGF-I and IR are similar in structure and both belong to the tyrosine kinase receptor family. They exist at the cell surface as hetero-tetramer receptors comprised of two α and two β subunits connected by disulfide bonds. The IGF-I/IR hybrid receptor consists of IGF-I α, β and insulin α, β hemi-molecules. The binding affinity of IGF-I/insulin/ IGF-II to each of the four receptors vary from each other, as illustrated, (adapted from (Hawkes and Kar, 2004))

The IGF-IR is thought to mediate mitogenic effects whilst the IR has more metabolic actions, although each can exert similar effects depending upon the context (Lu *et al.*, 2001) Each receptor has higher affinity for their corresponding ligand, additionally they bind with lower affinity to the other ligands. IGF-II can bind to the IGF-IR (6-fold lower affinity than

IGF-I) and to IR with different affinities based on the isoform of the IR present (Werner, Weinstein and Bentov, 2008).

Activation of the IGF-IR and the IR leads to phosphorylation of two different pathways. Ligand binding via initial autophosphorylation, leads to multiple substrate molecules like IRS-1, 2 and Src homology 2/collagen alpha proteins (SHC) to bind, and then activate intracellular signalling cascades: the PI3K pathway, which mainly activates metabolic activity (Ahmad, Singh and Glazer, 1999), and the MAPK pathway, which involved in cellular growth and differentiation (Pouysségur, Volmat and Lenormand, 2002), as illustrated in Figure 1.3.

PI3K activation results in the conversion of phosphatidylinositol 4,5 bisphosphate (PIP2) to phosphatidylinositol 3,4,4-triphosphate (PIP3), resulting in the activation of protein kinase B (AKT) causing an elevation of glucose uptake. Additionally, activation of the PI3K pathway promotes phosphorylation of 4E-binding protein 1(4EBP-1), resulting in the release of eukaryotic translation initiation factor 4E (eIF4E) and enhancing protein synthesis (Morita *et al.*, 2015). In addition, mTOR leads to ribosomal protein S6 kinase (S6Ks) phosphorylation, which promotes protein synthesis, and activates anti-apoptotic transcription factors causing lower cellular apoptosis (Morita *et al.*, 2015).

Conversely, the MAPK pathway is correlated with Src homology and collagen protein (SHC) phosphorylation, and the initiation of the growth factor receptor-bound protein 2 (GRB2) and mammalian son-of-sevenless (SOS) complex that activates Ras, MAP kinases and Like-1 protein (ELK1) leading to mitogenic signalling and regulation of apoptosis (Boney *et al.*, 2000), as illustrated in Figure 1.3.

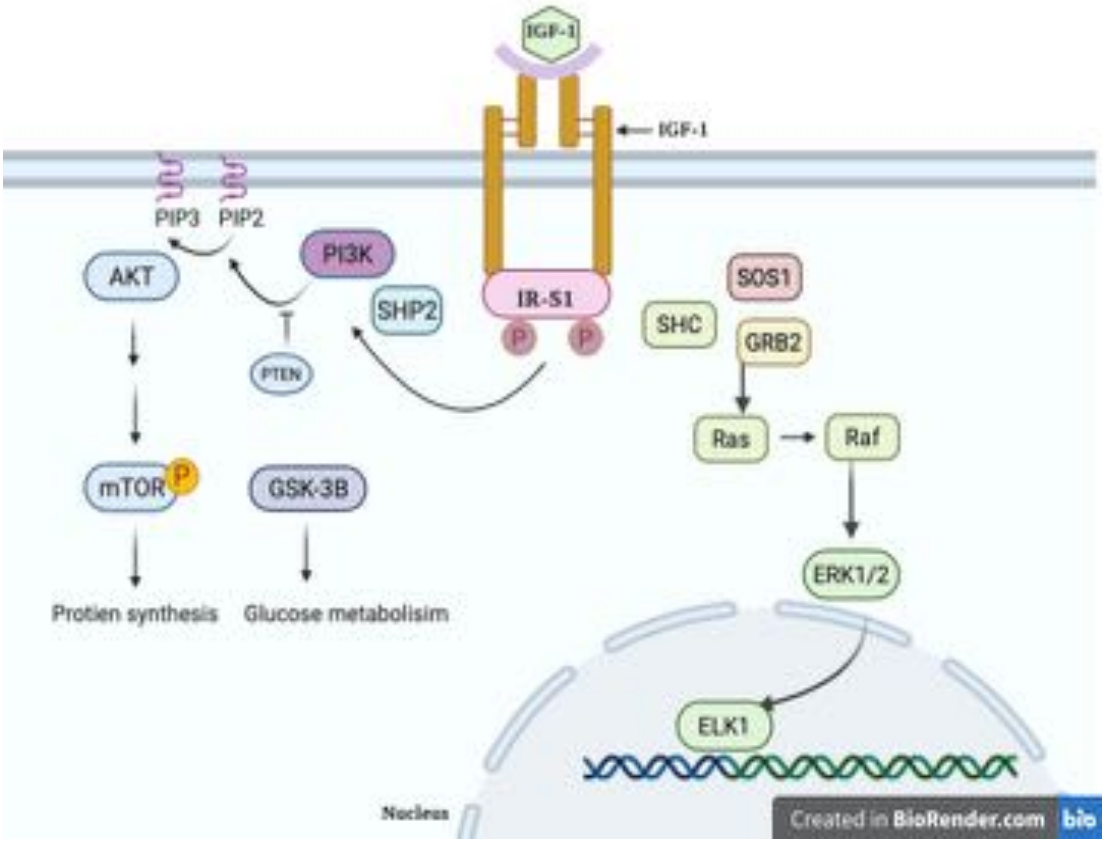


Figure 1. 4: The signalling pathways of the IGF-IR and IR (adapted from (Pollak, Schernhammer and Hankinson, 2004a)**):**
Ligand attachment is linked with activation of two downstream signalling pathways; PI3K pathway (left) which participates in metabolic functions, and the MAPK pathway (right) which participates in growth related functions.

1.3.3 IGF axis and breast cancer

Recent epidemiological study indicates that the association between overweight individuals, people with type 2 diabetes and high risk of different kinds of cancer mortality, when compared with those of a normal body weight who are not diabetic (Frystyk, 2004). Insulin resistance increases in metabolic tissues like fat, liver and muscle, due to obesity or

type 2 diabetes. Therefore, insulin resistance and a compensatory hyperinsulinemia develops. Consequently, insulin indirectly increases hepatic IGF-I production and the quantity of bioavailable IGF-I, in a direct or indirect action via reducing the levels of IGFBPs -1 and -2. Due to the poor levels of binding proteins, there is more free IGF-I to bind to the IGF-IR (Coughlin *et al.*, 2004; Gallagher and LeRoith, 2011).

In vitro studies have reported that the IGF-IR is necessary for the expression of genes that modulate tumour cell survival, the cell cycle, attachment, motility and metastasis (Lopez and Hanahan, 2002; Yakar, LeRoith and Brodt, 2005a). In breast tumour, the IGF-IR expression is frequently high and can play additional roles in cell transformation, cancer cell growth and metastasis, while reducing IGF-IR expression, results in reduced cancer growth in the majority of tumours (Lopez and Hanahan, 2002; Loughran *et al.*, 2005). Furthermore, high expression of the IGF-IR appears to be associated with radiotherapy resistance and lower patient survival (Jones *et al.*, 2009). It has been shown that the IGF system increases breast cancer growth and survival through different signalling cascades, such as PI3K/AKT and MAPK (Martin *et al.*, 2012). Furthermore, ERK (MAPK44/42) plays a crucial effect in the resistance of ER-positive , MCF-7, breast cancer cells, to cell apoptosis, that suggests the significance of ERK cascade in the continued survival of breast tumour (Kang, 2010). IGF-1R knockout mice exhibit a decreased rate of cancer proliferation and migration, suggesting a major effect of the IGF-IR in the growth of breast cancer cell (Yakar, LeRoith and Brodt, 2005b).

IGFBPs play an important role in the regulation of IGF-I bioavailability and may promote the effects of IGF-1 signalling in the tumour. It has been shown that both endogenous and exogenous IGFBP-1 can inhibit IGF-I induced proliferation and motility of breast cancers

in vitro and in athymic mice (Sachdev and Yee, 2007). On the other hand, IGFBP-2 is reported to function with oncogenic activities in breast cancer (Pickard and McCance, 2015), and may be involved in breast cancer metastasis.

Therefore, understanding the molecular mechanisms that lead to increases in the level of IGF-I signalling may reveal novel targets to improve therapeutic benefit and prevent breast cancer.

Patients with TNBC have a much poorer outcome in relation to other sub-types of breast cancer principally because there is no specific target for therapy. Roughly 41–46% of TNBC individuals express the IGF-IR (Bahhnassy *et al.*, 2015). Recent evidence indicates that IGF-I/IGF-IR level may be associated with TNBC patients and also with lower response and outcome, decreased survival and higher recurrence (Hartog *et al.*, 2011). It was suggested that targeting IGF-I signalling in TNBC may serve as a promising therapy (Bahhnassy *et al.*, 2015). A recent study has indicated that inhibiting the IGF-IR by using OSI-906 (Linsitinib) can increase the sensitivity of TNBC cells to PI3K inhibition and that IGFBP-3 levels may be used as predictor for sensitivity to PI3K/IGF-IR inhibitor therapy (De Lint *et al.*, 2016). It has been found that IGF gene was increased in TNBC cell lines and xenografts, and anti IGF-IR/IR therapy (inhibitor BMS-754807) in combination with chemotherapy, docetaxel, significantly increased TNBC cell sensitivity to chemotherapy (Litzenburger *et al.*, 2011).

1.4 Risk factors of breast cancer

1. Genetic predisposition

Roughly 10% of diagnosed breast tumours are correlated with a family history and inherited disease (Shiovitz and Korde, 2015). Some studies suggest that breast cancer can be caused by two high penetrance cancer suppressor genes, *BRCA1* and BRCA2, that normally function as tumour-suppressor genes and participate in DNA damage recombination and repair, apoptosis, cell-cycle checkpoint control and transcriptional regulation of DNA damage (Dine and Deng, 2013). A meta-analysis in in women aged 70 years or older proposed that the breast cancer risk ratio carrying *BRCA1* was 57% or *BRCA2* genes was 49% (Chen and Parmigiani, 2007). The prevalence of *BRCA1/BRCA2* genes differs between race groups, being less in Asian (0.5%) and high in Ashkenazi (10.2%) Jewish women (Daly *et al.*, 2016).

2. Lifestyle and other environmental factors

Women who have first-degree relative diagnosed with breast tumour can raise the risk of obtaining the disease (Feng *et al.*, 2018). The risk of breast tumours also raises with age due to the accumulation of random mutations in breast cells, which results in their transformation into cancer cells if/when sufficient mutations accumulate (Sun *et al.*, 2017) and the environment is conducive. Furthermore, it has been shown that early pregnancies and high levels of estrogen midst pregnancy decrease breast tumour risk (Britt, Ashworth and Smalley, 2007).

Other risk factors for breast cancer include obesity (Mcdonnell *et al.*, 2014), increased alcohol consumption (Chen *et al.*, 2011), smoking, prolonged use of hormonal

contraceptives or hormone replacement therapy (Mørch *et al.*, 2017; Busund *et al.*, 2018), and late menopause (Harbeck *et al.*, 2019).

3. Obesity

Obesity is an important risk factors for cancers, including breast. In the USA, overweight among women may cause 20% of cancer deaths and 14% of cancer deaths in men (He and Nelson, 2017). Evidence supports a link between obesity and insulin resistance with increased probability of various cancers including breast cancer (Martinez-Outschoorn *et al.*, 2017; Picon-Ruiz *et al.*, 2017). An epidemiological study by the International Agency for Research on Cancer (IARC) Working Group, found that overweight is linked with an increased risk of cancer for at the minimum 13 types of cancer, including breast (Lauby-Secretan *et al.*, 2016).

The biological correlation between obesity and cancer risk, may be linked to tissue lipid metabolism and circulating lipid levels (Korea *et al.*, 2014). Cancer cells demonstrate alterations in several conditions of lipid metabolism, which can promote the biological availability of lipids for the contribution of lipid to energy homeostasis, the synthesis of membranes and lipid signalling pathway functions, such as the activation of inflammation-related signalling cascades. These alterations are correlated to substantial cellular processes, such as cell differentiation, growth, and motility (Korea *et al.*, 2014).

Moreover, a meta-analysis study reported that increased daily fat intake is associated with increased risk of breast cancer (Mourouti *et al.*, 2015). There is a strong association between obesity and breast cancer due to increases in the rate of conversion of androgenic

precursors to estrogen through high aromatase activity in adipose tissue (Matthews and Thompson, 2016). Specifically, obesity raises the risk by 50% in post-menopausal women with ER-positive breast tumour (Dalamaga, Christodoulatos and Mantzoros, 2018).

Increased body weight and excess fat tissue are factors that contribute to epigenetic changes that affect gene expression. These can mediate several actions such as an increase in adipose tissue stores, which can lead to fat accumulation as ectopic fat tissue leading to increased risk for several diseases (Avgerinos *et al.*, 2019). Ectopic fat deposition is defined as the pathological growth of white adipose tissue in areas where it should not be (e.g. intra-abdominally, intrahepatic, intramyocellular, etc.). Also, fat accumulation may influence different pathways such as inflammatory, metabolic, and immunologic alterations changing gene function, deoxyribonucleic acid (DNA) repair and cell mutation rate, along with epigenetic changes allowing tumour progression and transformation (Avgerinos *et al.*, 2019).

The association of high levels of insulin and insulin-like growth factors in response to obesity can promote the proliferation of breast cancer cells (Picon-Ruiz *et al.*, 2017). The main regulator of glucose homeostasis is insulin. Chronic obesity can lead to insulin resistance due to hyperinsulinemia (Kahn and Flier, 2000).

IGFs synthesized by almost every tissue in the body, are important for development, proliferation, and survival, being associated with tumour pathogenesis (Moschos and Mantzoros, 2002).

Although, the role of obesity in cancer is not fully illuminated, the main pathways that link obesity to cancer include abnormalities in the IGF axis and hyperinsulinemia/IR signalling (Crowe *et al.*, 2011). In a cohort of 512 women who had early stage breast tumour, high

levels of fasting insulin were correlated with lower outcome and mortality (Goodwin *et al.*, 2002).

Cells utilise glucose for energy, which is required for growth and maintenance in normal and cancer cells. In the presence of oxygen, under normal conditions cells metabolise glucose to pyruvate via glycolysis, then pyruvate is converted into acetyl-coenzyme A (Acetyl-coA) in the mitochondria which is catalysed by the tricarboxylic acid (TCA) cycle and then enters oxidative phosphorylation (OXPHOS), which leads to the net production of 36-ATPs compared with 2-ATPs released by glycolysis alone (Zheng, 2012). However, a general phenomenon of tumours, is increased glucose catabolism via glycolysis into lactate even under normal oxygen conditions, instead of maximising ATP generation through OXPHOS. This phenomenon was first observed by Otto Warburg, and has since been termed the Warburg effect (Person, 1957) Despite early preconceptions tumour cells do not completely bypass the TCA cycle, particularly those with tumour suppressor gene expression and dysregulated oncogenes, but also rely on the TCA cycle which is supported by functional mitochondrial activity in cancer cells (Anderson et al., 2018). Although glycolysis produces less ATP than OXPHOS, the rate of ATP generation by glycolysis is much higher than by OXPHOS, which is more appropriate to the energy demands of malignant cells (Pfeiffer, Schuster and Bonhoeffer, 2001). Enhanced glycolysis also provides tumour cells with biochemical precursors needed for the synthesis of larger molecules such as proteins, lipids, and nucleic acids (Heiden, Cantley and Thompson, 2009). This remodeling of cell metabolism in breast tumours is mediated through the activation of oncogenes and tumour suppressor genes that cause changes to multiple intracellular signalling pathways that regulate enzymes of glycolysis synthesis, fatty acid

biosynthesis, protein synthesis and the pentose phosphate pathway (PPP) (Cairns, Harris and Mak, 2011). Obesity is related to essential metabolic alterations that enhance tumor growth, such as adipose tissue dysfunction, high blood levels of insulin and IGF-I, inflammation, and nutrient availability (Fallone *et al.*, 2018). These studies illustrate the mechanisms whereby obesity helps tumour cell proliferation (Vernieri *et al.*, 2016).

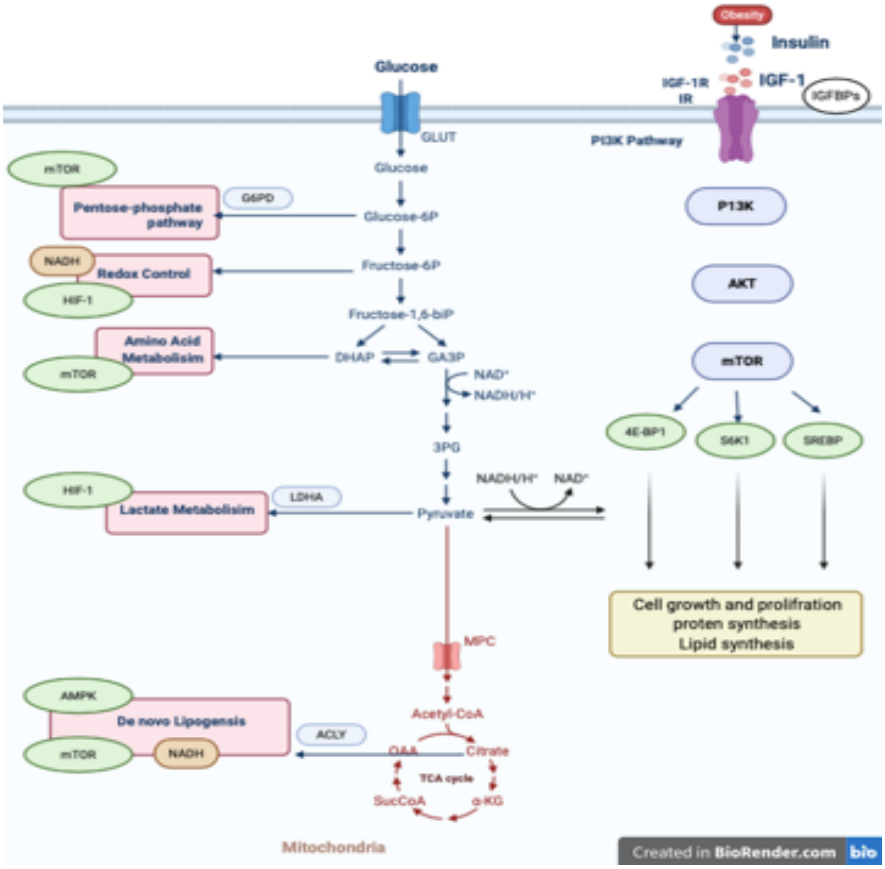


Figure 1. 5: Modulation of cancer metabolism by obesity-related growth factors.
Insulin and IGF-I promote the PI3K signalling pathway, which in turn upregulates several pathways such as glycolysis and metabolic to produce energy which can achieve the biosynthetic demands of growth. (IGFBPs) IGF-binding proteins, (IR) insulin receptor, (IGF-IR) IGF-I receptor, (AMPK) AMP-activated protein kinase, (HIF-1$^{\alpha}$) hypoxia-inducible factor 1-alpha, (SREBP) sterol regulatory element-binding protein, (mTOR) mammalian target of rapamycin, (OAA) oxaloacetate, (GSR) glutathione reductase, , (G6PD) glucose-6-phosphate dehydrogenase, , (ACLY) ATP citrate lyase, (GSX) glutathione peroxidase, (LDHA) lactate dehydrogenase A (ACC) acetyl-CoA carboxylase, (FAS) fatty acid synthase, (HK2) hexokinase 2, (ECM) extracellular matrix, (PFKFB3) 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3, (PEP) phosphoenolpyruvate,(PKM2) pyruvate kinase M2, (PDH) pyruvate dehydrogenase, (PFK1) phosphofructokinase-1, (CS) citrate synthase, (DCA) dichloroacetate (PDK) pyruvate dehydrogenase kinase.

1.5 Cholesterol and breast cancer pathogenesis

Recently, altered cholesterol metabolism, was positively and independently correlated with risk factors for breast tumour in post-menopausal women (Mcdonnell *et al.*, 2014). Data from the Canadian National Cancer Surveillance System study states that postmenopausal women with high cholesterol levels had a 48% increase in the risk of breast tumour (Danilo and Frank, 2012). Investigating the biological mechanisms by which high cholesterol metabolism impacts on breast cancer risk and progression may lead to novel therapeutic targets.

1.5.1 Cholesterol function and metabolism

Diet is a confirmed risk factor for different cancers. Several mechanisms have been investigated to underlie the link between different dietary patterns and the risk of cancer, including epigenetics, gut microbiota and their metabolites, immune function, inflammation, and metabolic or hormonal disruption (Cedó *et al.*, 2019). Recent studies indicate a positive association between high fat and cholesterol diets and cancer progression and metastasis(Pelton *et al.*, 2014).

Cholesterol is an essential molecule, that maintains the integrity and fluidity of the cell and organelle membranes. It plays an important role in maintaining cell homeostasis and acts as a building block for steroid hormones, oxysterols, bile acids and vitamin D (Hu *et al.*, 2012). Cholesterol is insoluble in plasma, and requires transporters known as lipoproteins, which are made up of a hydrophobic core, containing a hydrophilic coat, cholesteryl esters and triglycerides consisting of free low-density lipoprotein (LDL) cholesterol, phospholipids and apolipoproteins, as outlined in fig 1.5. There are several categories of lipoprotein: LDL, high density (HDL), intermediate density (IDL), ultra-low density

(ULDL, also known as chylomicrons) and very low density (VLDL) (Röhrl and Stangl, 2018).

The main cholesterol-carrying lipoproteins are LDL and HDL (Röhrl and Stangl, 2018). Therefore, excess cholesterol from tissues and peripheral cells is transferred directly to HDL, which accepts excess cholesterol and transports it back to the liver for disposal in bile acids. This pathway ensures that cholesterol is removed from the body by the gallbladder or redistributed to other tissues, as outlined in figure 1.5 (Shahoei and Nelson, 2019).

All cells can synthesize cholesterol, and 50% of total cholesterol synthesis in humans body occurs in the liver (Luo, Yang and Song, 2020) by highly orchestrated cholesterol transport molecules that release and absorb cholesterol from the diet (Azrolan and Coleman, 1989). Indeed, the rate limiting step for the regulation of the cholesterol biosynthetic pathway is the endoplasmic reticulum (ER) enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), that is responsible for converting the acetyl-CoA to HMG-CoA to mevalonate. The latter is converted to iso-pentenyl pyrophosphate, next converted to farnesyl-pyrophosphate (farnesyl-PP), which is modified to form cholesterol, as outlined in figure, 1.6 (Baek and Nelson, 2016). The accumulation of cholesterol lowers the activity of HMG-CoA reductase and a variety of other enzymes, thereby limiting the production of cholesterol via the biosynthetic pathway (Goldstein and Grown, 1997).

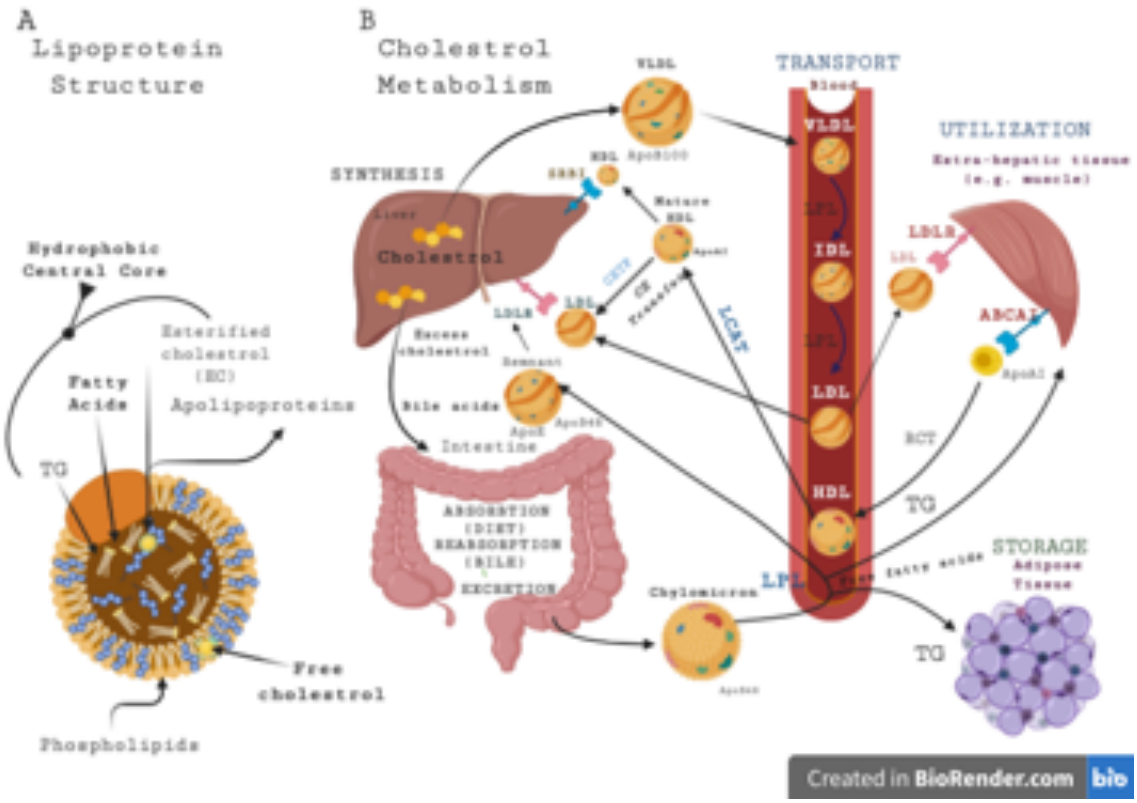


Figure 1. 6: Describe A) lipoprotein structure and B) cholesterol metabolism and transport, adapted from (Leon *et al*., 2015).

The lipoprotein consists of triglyceride (TG), free cholesterol (FC), free fatty acids FFA, and phospholipid (PL). B) cholesterol synthesis occurs in hepatocytes and is transported by two ways: first as VLDL to the blood circulation and the VLDL contain fatty acids, TG, free cholesterol and phospholipids. VLDL particles are transported by apolipoproteins. Then, VLDL is remodeled and transformed into IDL and LDL by the action of LPL in the blood circulation. Second way to transport cholesterol, is transported by chylomicrons from intestine to the circulation by the action of LPL. LDL particles are removed from blood stream through LDLR.

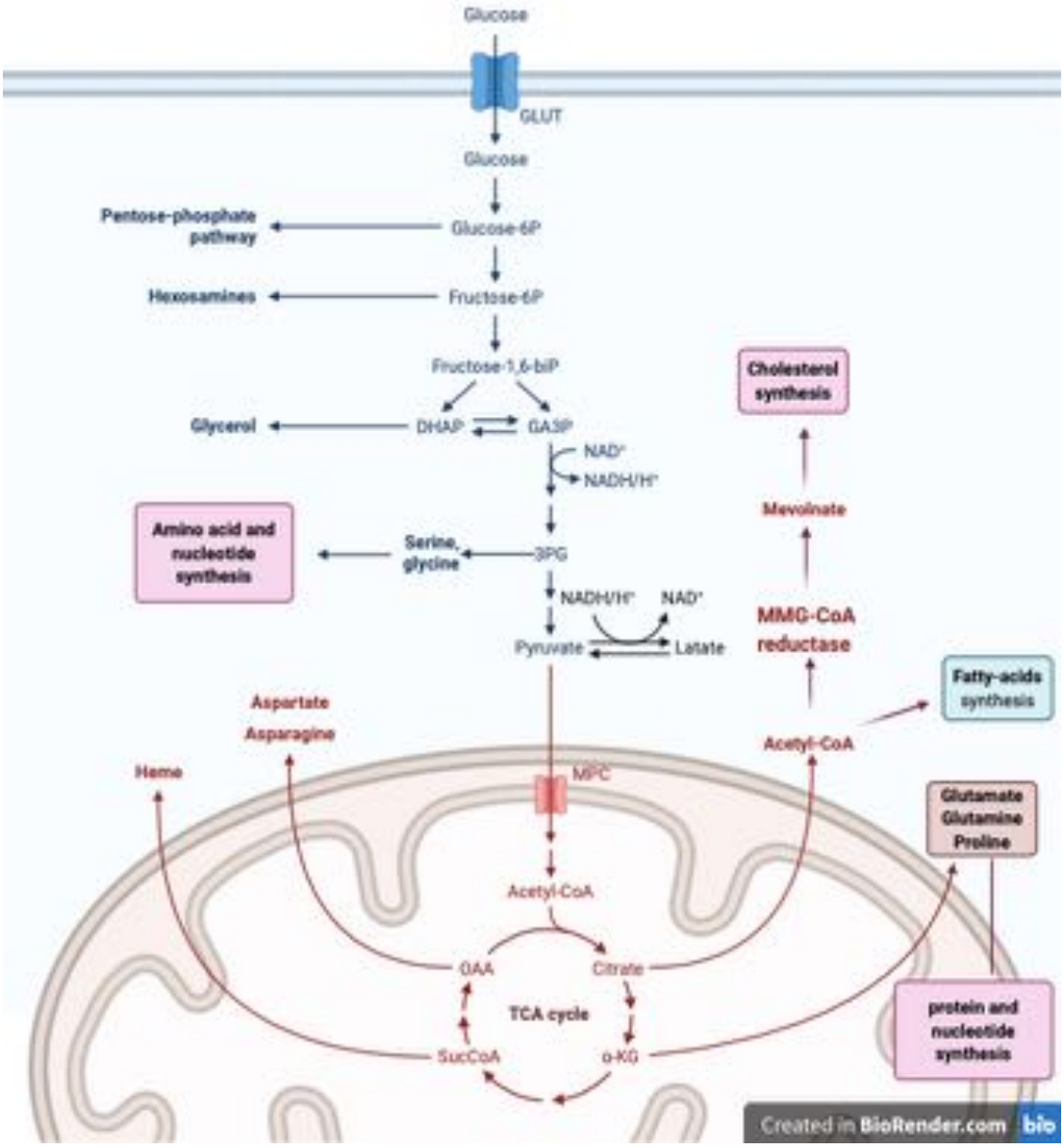


Figure 1. 7: Cholesterol biosynthesis pathway:
A biochemical signalling pathway demonstrating the key steps by which cholesterol is synthesised from acetyl-CoA. HMG-CoA reductase is the rate limiting step. adapted from (Vernieri et al., 2016).

1.5.2 Cholesterol processing

The bile acid synthesis pathway is the main pathway, in which cholesterol is converted into oxysterols through enzymatic and nonenzymatic pathways. The replacement pathway is the acidic bile acid synthesis pathway, identified by the hydroxylation of cholesterol by CYP27A1, producing 27-hydroxycholesterol (Brown and Jessup, 1999).

1.5.3 Regulation of intracellular cholesterol homeostasis

Homeostasis of intracellular cholesterol is controled by complex and highly regulated signalling cascades that depend on both short negative feed-back loops and longer loop feed-forward mechanisms (Goldstein and Grown, 1997). In brief, the level of intracellular cholesterol is maintained at a low level by being incorporated into membranes, being stored as a cholesterol-ester in the cytoplasm or being exported out of the cell (Chang *et al*., 2009). In an acute need for increased intracellular cholesterol, as happens during cell division, the cell depends on *de novo* synthesis, decreased efflux, and/or increased uptake of cholesterol (Baek and Nelson, 2016).

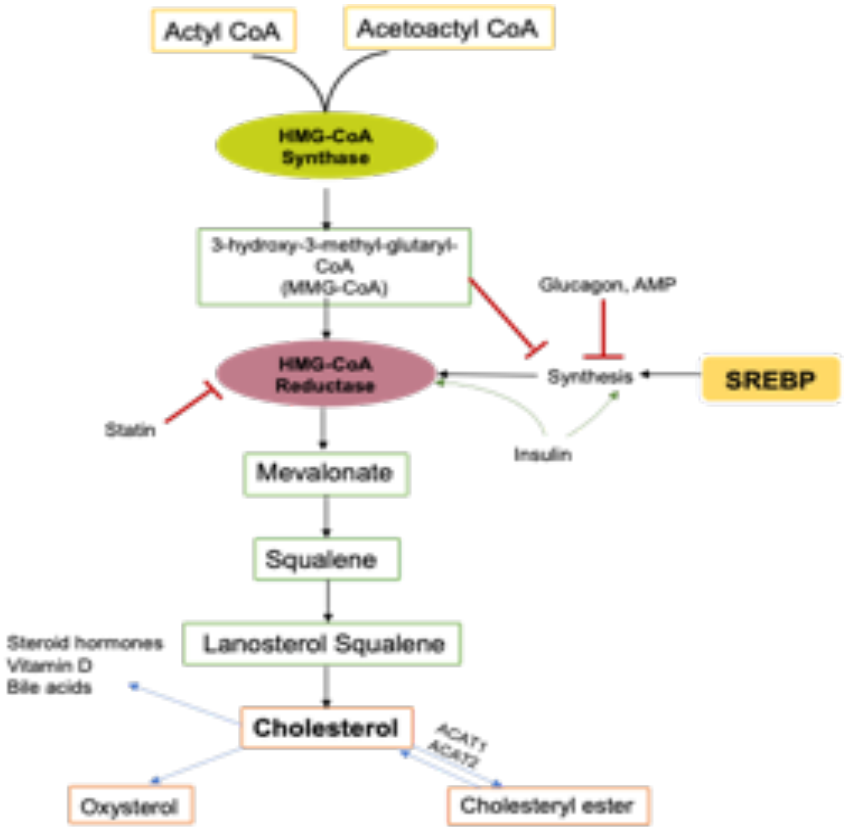


Figure 1. 8: Cholesterol biosynthesis pathway:
cholesterol biosynthesis (intracellular) is dysregulated in cancer cells due to increased acetyl CoA levels via glycolysis, fatty acid biosynthesis and HMG-CoA reductase activity, which is the major regulator of cholesterol homeostasis is sterol regulatory element-binding proteins-2 (SREBP2), adapted from (Huang, Song and Xu, 2020).

1.5.4 Role of cholesterol in the development of breast cancer

The link between a high cholesterol diet and the risk of breast cancer was first discovered in the 1950s. Circulating levels of cholesterol are approximately 453 µg/ml or less for healthy adults (Baek and Nelson, 2016). A meta-analysis proposed that dietary cholesterol intake increased the risk of breast cancer (Li *et al.*, 2016). Preclinical studies strongly demonstrate that cholesterol can influence cancer pathophysiology and is an important mediator of the effects of obesity (Ding *et al.*, 2019). Intracellular biosynthesis of

cholesterol occurs via the mevalonate pathway. Clinical and experimental evidence supports that cholesterol biosynthesis (intracellular) is dysregulated in many cancer cells due to increased acetyl CoA levels via glycolysis, fatty acid biosynthesis or HMG-CoA reductase activity (Kwan *et al.*, 2008; Ahern *et al.*, 2011), as described in figure 1.7.

An imbalance in cholesterol levels is triggered by increased food consumption or genetic factors for instance, ATP-binding cassette subfamily A member 1 (ABCA1) gene and other genes as myosin regulatory light chain interacting protein (MYLIP, also defined as IDOL), which leads to an accumulation of cholesterol in peripheral tissue (Hegele, 2009). Furthermore, it's a precursor to steroid hormones and bile acids, which can initiate or enhance breast cancer (Finlay-Schultz and Sartorius, 2015). Cholesterol is important for the fluidity and integrity of the cell and cellular organelle membranes, thus high levels of cholesterol are required for cells that have a high proliferative rate for membrane biogenesis. In addition, the cholesterol-derived metabolite 6-oxo-cholestan-3β,5α-diol, is found to be increased in patients with breast tumour, binds glucocorticoid receptors, the nuclear receptor of endogenous cortisol, and subsequently promotes cancer growth (Voisin *et al.*, 2017). In general, cholesterol metabolism associates with cancer growth, migration and invasion (Liu *et al.*, 2018; Chimento *et al.*, 2019a). An increase in cholesterol biosynthesis is one of the hallmarks of cancer. In particular, when oxygen and/or lipids are limited, for example in the glioblastoma microenvironment, the key transcription factor SREBP2 and its downstream targets genes, in addition to mevalonate-pathway enzymes, are highly upregulated in cancer, as outlined in graph 1.7 (Lewis *et al.*, 2015). Beside

SREBP, RAR-related orphan receptor gamma (RORγ), promotes the cholesterol-bio-synthesis pathway and mediates progression of TNBC (Cai *et al.*, 2019).

As a vital component of the cell membrane, cholesterol may be related to membrane receptors which can activate oncogenic signalling pathways such as MAPK (Chun Wang *et al.*, 2017) and Notch (Levental and Veatch, 2016; Kim, 2019). Thus, the activation of signalling pathways resulting in cell differentiation, cell proliferation and tumour formation (Ding *et al*.,2019). Cholesterol can activate the PI3K/Akt and Wnt/β-catenin pathways which are related to cell proliferation and tumour formation (Lee, Loh and Yap, 2015), and in prostate cancer cells it has been shown that the activation of PI3K/Akt leads to the accumulation of cholesterol esters through increasing LDL uptake and causing further esterification (Yue *et al.*, 2014). Besides the cell membrane, cholesterol can function in the cytoplasm. It has been shown that lysosomal cholesterol activates mTORC1, mammalian target of rapamycin complex 1, which is responsible for cell growth, invasion and metastasis of breast cancer cell lines (Ricoult *et al.*, 2016; Kim, Cook and Chen, 2017). It has been found that cholesterol accelerated renal cancer cell invasion and migration mediated through KLF5/miR-27a/FBXW7 pathway, which plays a crucial role in epithelial-mesenchymal-transition, chemoresistance, and regulating cancer immune response(Liu *et al.*, 2018).

In contrast, statin treatment inhibits *de novo* cholesterol synthesis and decreases tumour growth rates. A recent meta-analyses reported that targeting the mevalonate pathway by statins, specifically lipophilic statins show improved recurrent-free, overall survival in breast tumour patients (Manthravadi, Shrestha and Madhusudhana, 2016; B. Liu *et al.*, 2017; Chimento *et al.*, 2019b; Göbel *et al.*, 2020). Lipid rafts are an important component

of cell membranes that are rich in cholesterol sphingolipids. The function and structure of the lipid rafts depends on the composition of cholesterol and related phospholipids, which are platforms for cellular signal transduction (Chimento *et al.*, 2019a). Consequently, upregulation of cholesterol levels may lead to structural damage in lipid rafts, which may inhibit the function of raft-related proteins, such as tyrosine protein kinases (Src and c-Met,), death receptor proteins and calcium channels(Zeng *et al.*, 2018). Using a lipid raft inhibitor methyl-β-cyclodextrin (MβCD) reduced the migration of lung cancer cells by 63.1-83.3%, indicating that cholesterol reduction in lipid rafts could inhibit the phosphorylation of lipid-raft associated proteins, Src and c-Met which could lead to a reduction in tumour formation, migration and invasion (Zeng *et al.*, 2018). Clinical evidence also supports that cholesterol metabolism is important for cancer progression (Guan *et al.*, 2019). On the other hand, a cholesterol-rich diet in a mouse model of hepatocellular carcinoma, increased membrane cholesterol levels, and consequently enhanced CD44 , a gene on chromosome 11q13 that encodes cell-surface glycoprotein participated in cell–cell interactions and invasion, migration, relocation into membrane lipid rafts and decreased the interaction between CD44 isoforms and ezrin complex, which are important for cell migration and cancer metastasis (Yang *et al.*, 2018). This study suggests that high cholesterol level decrease hepatocellular carcinoma invasion and metastasis.

1.5.5 Epidemiological studies on cholesterol and breast cancer

LDL and HDL are the main transporters for cholesterol, and there are several clinical studies that associate high circulating levels of LDL with breast cancer risk (Guan *et al.*, 2019). A recent retrospective cohort study in breast cancer patients, reported that

treatment with adjuvant chemotherapy resulted in elevated levels of total cholesterol, LDL-C, triglycerides and Apo B, and a decrease in Apo A1 and HDL-C (Li *et al.*, 2018). Additionally, a systemic LDL-C level of more than 117 mg dL−1 was proposed to be a prognostic factor of cancer stage, and it was significantly correlated with worse prognosis due to a higher proliferative rate, a higher histological grade, and a more advanced clinical stage. Also, patients with high LDL-C <144 mg dL−1 were found to have lymph node metastases (Rodrigues dos Santos *et al.*, 2014).

1.6 Low density lipoprotein (LDL)

1.6.1 The effects of LDL-cholesterol on breast cancer progression

LDL-c is frequently up-regulated in obese women (Luo *et al.*, 2014). Treatment with LDL-cholesterol (100ug/ml) for 48 hours *in vitro* was found to increase breast cancer cell proliferation, migration and loss of adhesion (Rodrigues Dos Santos *et al.*, 2014), Using *in vivo* mouse models of breast cancer, LDL-c treatment was shown to increase breast cancer growth together with a higher frequency of lung metastasis compared to untreated conditions (Rodrigues dos Santos *et al.*, 2014). Fast proliferating breast tumour cells have an increased requirement for cholesterol and this is met via increased uptake of LDL-c from the blood stream in breast cancer tissue through increased LDL-R expression (Pires *et al.*, 2012). LDLR gene mutation and protein expression is found to be high in ER-negative, MDA-MB-231, versus ER-positive, MCF-7, breast cancer cells (Cedó *et al.*, 2019). In ER-negative breast cancer cells LDL increased their growth (Gallagher *et al.*, 2017a), and migration (Gallagher *et al.*, 2017a) but not in ER-positive. This demonstrated that the difference between ER- negative and- positive breast cancer cells is related to

higher abilities of TNBC to store, take up, and utilise exogenous free cholesterol, and this was thought to be associated with the high activity of acyl-CoA:cholesterol acyltransferase 1 (ACAT1) (Antalis *et al.*, 2011). LDL-c promoted the proliferation of ER-positive breast cancer cells BT-474, because BT-474 also overexpress the HER2 (ErbB2) receptor (dos Santos *et al.*, 2014). Furthermore, it has been reported that high plasma levels of LDL-C were related to HER2-positive breast tumours (Rodrigues Dos Santos *et al.*, 2014). Several studies have explained the association between LDL and cancer to be due to LDL induced phenotypic changes in ERK/MAPK (Danilo *et al.*, 2013), PI3K/AKT (Khaidakov and Mehta, 2012; Alikhani *et al.*, 2013), JNK and ErbB2, down-regulation of adhesion molecules cadherin-related family member3, CD226, claudin7 (Rodrigues dos Santos *et al.*, 2014) and integrin β receptors (β1, β2 and β3)(Pan *et al.*, 2012). The PI3K/Akt pathway has also been identified as a mediator of the tumour inducing activity of hypercholesterolemia in breast cancer (dos Santos *et al.*, 2014).

A high-fat/high-cholesterol (HFHC) diet in a mouse model of breast cancer (MMTV polyoma middle T (PyMT) oncogene transgenic mice) was shown to enhance tumour progression (Cedó *et al.*, 2019). Another study showed that a HFHC diet significantly increased circulating cholesterol levels, induced angiogenesis and the growth of ER-negative (MDA-MB-231) human breast cancer cells in an orthotopic mouse model of breast cancer (Llaverias *et al.*, 2011).

1.7 Oxysterols and cholesterol metabolism

Cholesterol molecules generate oxidized metabolites, defined cholesterol oxidation products (COP) or oxysterols (Pelton *et al.*, 2014). Oxysterols can be triggered by

enzymatic or non-enzymatic oxidative processes, such as 24-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol (27OHC), or other products 6-hydroxycholesterol , 7α/β-hydroxycholesterol and 7-ketocholesterol, and act by binding nuclear receptors such as liver X receptor (LXR) and related orphan receptor (ROR) as described in figure 1.8 (Kloudova, Guengerich and Soucek, 2017). At a physiological concentration, oxysterols are natural constituents of the human body and modulate several important functions. The regulation of cholesterol synthesis is mediated by both oxysterols and cholesterol, as described in figure 1.9. Oxysterols affect different signalling cascades, membrane fluidity, and the activity of several membrane proteins (Fig. 1.9)(Kloudova, Guengerich and Soucek, 2017). Several clinical and experimental studies have suggested a role for 27OHC in cancer (Nelson, 2018; Jiao *et al.*, 2020).

The sterol regulatory element-binding proteins (SREBPs) function as major regulators of cholesterol biosynthesis and regulate the genes encoding cholesterol synthesis enzymes (Horton, Goldstein and Brown, 2002). SREBPs enhance the transcription of cholesterol-associated molecules, down-regulating genes that excrete or catabolize cholesterol and upregulating cholesterol synthesis genes. They are also associated with cholesterol uptake for example LDLR (Horton, Goldstein and Brown, 2002). SREBPs regulate cholesterol synthesis: when cellular cholesterol is high, SREBPs are found in the membrane of the ER where they produce a complex with SREBP-cleavage stimulating protein (SCAP) (Brown, Radhakrishnan and Goldstein, 2018). On the other hand, when the cellular cholesterol is low, the SREBP–SCAP complex moves to the Golgi apparatus, where it is cleaved and SREBP goes to the nucleus and activates the expression of associated genes, such as, insulin-induced gene (Insig) proteins (LP *et al.*, 2005a). The SREBP–SCAP compound

persists in the ER. However, cholesterol and oxysterols together regulate activation of SREBP: oxysterol by interaction with Insig protein and cholesterol through binding together with SCAP (Kloudova, Guengerich and Soucek, 2017).

Cholesterol metabolism is also modulated via HMGCR, a major enzyme of the cholesterol biosynthesis pathway. HMGCR has a sterol-sensing domain and therefore directly bind to the Insig protein, inducing degradation of the HMGCR (Gill, Chow and Brown, 2008).

Cholesterol levels are also regulated by LXRs, which can activate gene expression of sterol transporters, ATP-binding cassette subfamily A member 1 (ABCA1). In addition, oxysterol-binding proteins (OSBPs) participate in the downregulation of the ABCA1 transporter via modulation of protein destabilization and gene expression (Gill, Chow and Brown, 2008).

1.7.1 27-Hydroxycholesterol

The most abundant oxysterol in human plasma is 27OHC, which in human plasma ranges from 0.2-0.8 μM. High circulating levels of 27OHC are associated with hypercholesterolemia, and with increasing age (Russell, 2000). 27OHC is a selective estrogen receptor modulator (SERM) and is also a modulator of LXR. In an acidic pathway through hydroxylation, 27OHC is synthesised from cholesterol by the mitochondrial resident cytochrome P450 enzyme, CYP27A1 (sterol 27 hydroxylase, cholesterol 27-hydroxylase) (Clai *et al.*, 1991).

CYP27A1 is a ubiquitous mitochondrial enzyme, overexpressed in the liver, and expressed in the intestine, macrophages, brain and vasculature. 27OHC is principally transported, primarily in the esterified form, in association with LDL and HDL (He and Nelson, 2017).

27OHC catabolism occurs when 27OHC is hydroxylated by CYP7B1 oxysterol 7α-hydroxylase, which is expressed in the liver (He and Nelson, 2017).

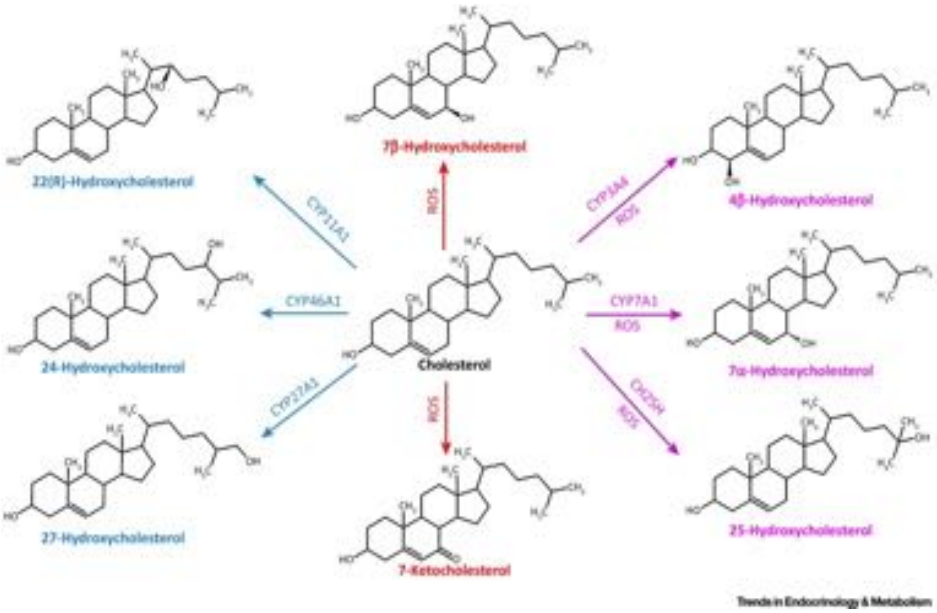


Figure 1.9: Cholesterol oxidation products;
generated by both non-enzymatic and enzymatic reactions. The enzymatic pathway is modulated by enzymes from the cholesterol-25-hydroxylase (CH25H) and cytochrome P450 (CYP) family.

1.7.2 27-Hydroxycholesterol metabolism

Oxysterols, modulate several different signal transduction pathways that regulate many biochemical and physiological effects, as shown in (fig 1.9). The bioavailability of 27OHC at the tumour site can be totally different from its level in the plasma, and this may be more critical in the context of tumour progression. The content of 27OHC in ER-positive breast cancer cells is 2.5- 3-fold higher than in normal breast cells (Wu *et al.*, 2013). At very low concentrations, oxysterols play a vital role in the human body, mediating many

physiological functions; they regulate cholesterol metabolism, influence signalling molecules such as MAPK, insulin-induced gene (INSIG); SREBP- SCAP cleavage activating protein, and the activity of some membrane proteins such as ABCA1, ABCB1, Na+/ K+ ATPase and also affect membrane fluidity (fig. 1.9) (Russell, 2000). However, oxysterol metabolism is also connected with different human diseases, like Parkinson's disease, Alzheimer's, and atherosclerosis. Recent evidence shows that oxysterols influence cancer progression and carcinogenesis (Kloudova, Guengerich and Soucek, 2017).

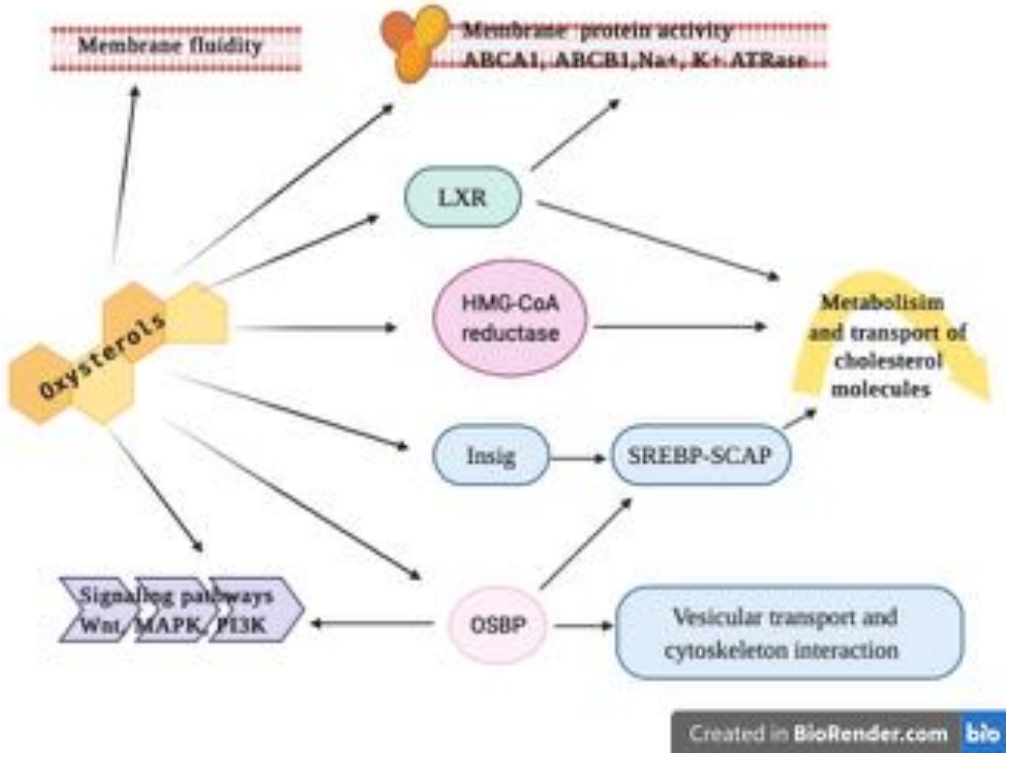


Figure 1. 10: The physiological role of oxysterols in cellular processes:
Oxysterols mediate many physiological functions; they regulate cholesterol metabolism, influence signalling molecules such as MAPK, INSIG, SREBP- SCAP, and the activity of some membrane proteins such as ABCA1, ABCB1, Na+/ K+ ATPase and affect membrane fluidity, adapted from (Russell, 2000).

1.7.3 27-Hydroxycholesterol and breast cancer

Oxysterols may modify cancer risk in different ways. There are several studies indicating the proliferative and the pro-cancerous role of oxysterols (Nelson *et al.*, 2013b; Raza *et al.*, 2017) , which could explain observations such as, higher circulating 27OHC correlated with aggressive cancer biology and late lethal disease of ER-positive breast cancer in postmenopausal women (Kimbung *et al.*, 2020) (Fig 1.10). Many studies investigated the mechanisms involved in the effects of 27OHC on promoting breast cancer development. A role for 27OHC in the regulation of p53 activity and proliferation has been reported via the E3 ubiquitin ligase Mouse Double Minute 2 protein (MDM2) in ER-positive (MCF-7) breast cancer cells. P53 is a tumour suppressor protein which plays a crucial role in apoptosis, senescence, and cell cycle. Also, the level of p53 is regulated by MDM2. However, 27OHC-induced an increase in the p53 regulator MDM2 and activates the interaction between p53 and MDM2. This suggests that 27OHC increased the proliferation through MDM2-mediated p53 degradation (Raza *et al.*, 2015). Another study investigated the role of 27OHC on MYC protein (a proto-oncogene and encodes a nuclear phosphoprotein) in MCF-7 breast cancer cells. MYC is a critical oncogene protein involved in the proliferation, migration and invasion of different types of cancers (Xu, Chen and Olopade, 2010). This study found that 27OHC increased MYC stability through transcriptional repression of protein phosphatase 2A (PP2A), F-box and WD repeat domain containing 7 (FBW7) and small C-terminal domain phosphatase 1(SCP1), in ER-positive breast cancer cells (Ma *et al.*, 2016).

As a result, Shen *et al* realized that 27OHC is a risk factor in both ER-positive and -negative breast cancer, especially in post-menopausal patients. (Shen *et al.*, 2017). Furthermore, it

has been shown that 27OHC promotes proinflammatory processes mediated through ERα (Umetani *et al.*, 2014); therefore, suggesting the link between 27OHC, inflammation and cancer development. However, the mechanisms involved in 27OHC enhanced development of breast cancer are complicated and require further study (Umetani *et al.*, 2014).

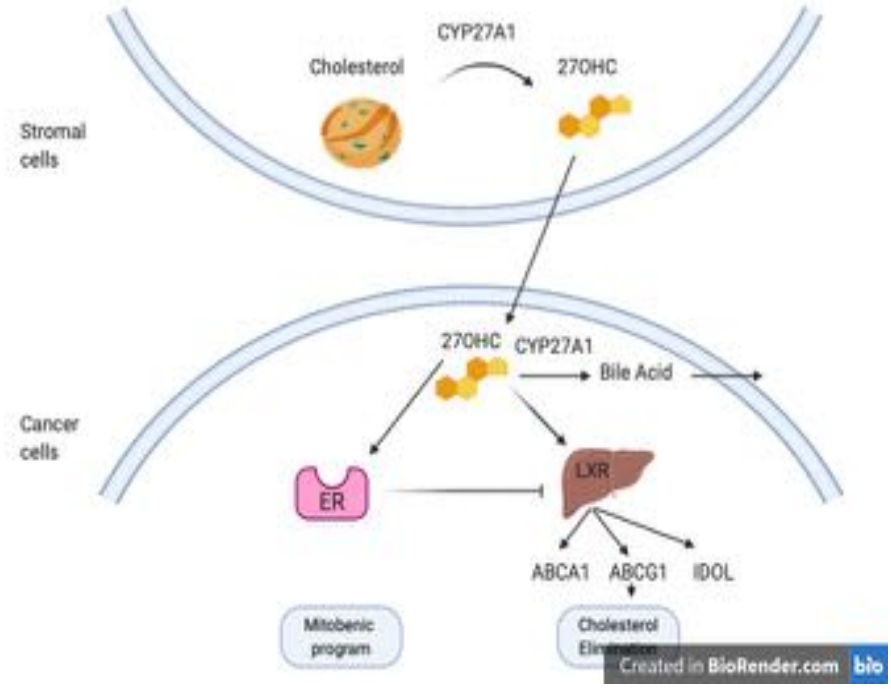


Figure 1. 11: The cholesterol metabolite 27OHC influences breast cancer cell biology as a modulator of the LXR and ER:
The cholesterol metabolite 27OHC promotes breast cancer cell progression via its actions on the LXRs and ER. CYP27A1, the enzyme that converts cholesterol to 27OHC, is highly found in macrophages, liver, and in stromal cells within breast cancer. Under normal physiologic circumstances, 27OHC might be converted to polar bile acids by the enzyme CYP7B1 and/or might play a role as an agonist ligand of LXR to regulate cholesterol homeostasis in cells. 27OHC can act through the ER and promote breast tumour proliferation, adapted from (Mcdonnell et al., 2014).

1.7.3.1 27-Hydroxycholesterol as a modulator of the estrogen receptor (ERα)

Since researchers discovered that 27OHC was a SERM of the ERα, a number of studies have described, that activation of the ER is increased by 27OHC in breast cancer (DuSell *et al.*, 2008; Umetani and Shaul, 2011). 27OHC is synthesised through CYP27A1 enzyme and catabolised by CYP7B1 into bile acid. SERMs are characterized as compounds that suppresses or stimulate ERα activity and their action is dependent on the tissue type (Wardell, Nelson and McDonnell, 2014) , as illustrated in figure 1.10. 27OHC levels are increased within breast cancer tissue compared to normal breast tissue, and this was correlated with a higher tumour grade, and circulating 27OHC levels were increased in women treated with an aromatase inhibitor (Nelson *et al.*, 2013b; Wu *et al.*, 2013), which culminates in increased cell proliferation and consequently promoted ER-positive breast cancer progression (DuSell *et al.*, 2008).

The exposure to 27OHC is correlated with the development of breast cancer and this action is mediated through the ER in a mouse model of breast cancer (Nelson *et al.*, 2013b).

The effect of 27OHC on the growth of MCF-7 cells at a concentration of 1–2 μM was comparable to that of 1–2 nM of 17β-estradiol, and they suggested that 27OHC promotes breast cancer growth through binding to ER (Cruz *et al.*, 2010). The proliferative role of 27OHC has been confirmed *in vitro*, MCF-7, and *in vivo*, PyMT mice and murine human cancer cell xenografts (Umetani and Shaul, 2011; Nelson *et al.*, 2013b).

Elevated 27OHC can increase the metastasis of breast cancer in murine models, and as such was able to promote the myeloid immune cell functions through the interaction with the number of γδ-T cells and polymorphonuclear-neutrophils cells, resulting in a decrease in number of cytotoxic $CD8^{+}$T cells, and this mechanism explained the effect of 27-

hydroxycholesterol on metastasis as this needs myeloid immune function (Baek *et al.*, 2017). Several clinical studies indicate that 27OHC works as a biochemical mediator of the metastatic impact of hypercholesterolemia, as 27OHC was found to increase markers of EMT and consequent lung metastases in MMTV-PyMT mice (Nelson *et al.*, 2013a; Wu *et al.*, 2013). Furthermore, 27OHC promoted invasion and migration in both ER-positive and ER-negative breast cancer cells through elevated EMT and activated, Signal transducer and activator of transcription 3, STAT-3 (Shen *et al.*, 2017). However, the discovery of 27OHC as a selective endogenous modulator of the ERα that promotes ER-positive breast cancer proliferation could help to explain why some breast cancer patients have resistance to aromatase inhibitors, where 27OHC, in a low-estrogen environment, may act as an alternate estrogenic ligand (Simigdala *et al.*, 2016).

1.7.3.2 27-Hydroxycholesterol as a modulator of the LXR

LXR is a group of proteins affected by the level of oxysterols in the plasma (called oxysterol receptors) – LXR-α and LXR-β form heterodimers with retinoid X receptors (RXR) (Wu *et al.*, 2016a). It has been demonstrated that LXR plays a significant role in ERα-positive breast cancer cells, such as arresting cell cycle progression and reducing cell proliferation (Wu *et al.*, 2016b). Moreover, 27OHC plays a role as a positive regulator of LXR activity in breast cancer (Bovenga, Sabbà and Moschetta, 2015). It has been demonstrated that the synthetic LXR agonist reduces cell proliferation but increases EMT markers in MMTV-PyMT mice, such as Vimentin, Snail Family Transcriptional Repressor 1(Snail1) and Fibroblast Activation Protein Alpha (FAPa) (Hassan *et al.*, 2015). A recent study illustrated that in an ER-independent and LXR-deficient breast cancer cell line, 27OHC increased migration and invasion. Additionally, they found that 27OHC increased

migration and invasion not only in ER-positive breast cancer cells but also ER-negative cell lines that involved STAT3 activation (Shen *et al.*, 2017). It has also been observed that 27OHC increased breast cancer proliferation through ER transcriptional activity and that the ER may inhibit the protective effects of LXR in ER-positive breast cancer (Nelson *et al.*, 2013a)

1.7.4 The impact of high cholesterol on the IGF axis

As a growth factor, IGF-I plays a key factor in lipid metabolism (Aguirre *et al*., 2016). IGF-I knockout mice exhibited dysregulated expression of genes encoding enzymes and lipid-related genes involved in cholesterol synthesis in the liver (Ita *et al*., 2015). Data from the large-scale, community based Framingham Heart Study, illustrated that lower IGF-I levels are correlated with metabolic syndrome (Lam *et al.*, 2010; Zelazowska-Rutkowska *et al.*, 2018). A recent cross-sectional study indicated that IGF-I levels associate with LDL-C levels in short-stature children and adolescents (Zhao *et al.*, 2019), and they suggested that IGF-I plays an important role in regulating the LDL-C concentration in serum. In oligodendrocyte progenitor cells, long-term suppression of cholesterol biosynthesis inhibited IGF-I stimulated AKT/PI3K phosphorylation and cell survival. They explained that adding 25hydroxycholesterol to cells in culture inhibited cholesterol synthesis which then depleted lipid rafts and this prevented IGF-I activation of the IGF-IR (Romanelli *et al.*, 2009). Additionally, IGF-1 induces different expression of fatty acid and cholesterol biosynthesis signalling genes in murine C2C12 myoblasts (muscle cells) which was explained by IGF-1 treatment upregulating 19 genes that are correlated with cholesterol synthesis and five genes with fatty acid biosynthesis and they suggested that IGF-I induces the expression of cholesterol and fatty acid biosynthesis through the activation of the

SREBP transcription factor (Bhasker and Friedmann, 2008). Depletion of cellular cholesterol also impacts on the pathways activated by IGF and EGF, through blocking activation of the PI3K/AKT pathway. Cholesterol lowering treatment inhibits the IGF-IR in bile duct cancer cells (Laurentiis, Donovan and Arcaro, 2007; Lee *et al.*, 2016).

1.8 The role of cholesterol-lowering treatments on breast cancer

Studies have concluded that cholesterol and its major metabolite, 27OHC, may promote breast tumour progression, development and metastasis and in support of this several studies have shown that cholesterol-lowering drugs may be a potential therapy to reduce cholesterol and reduce the risk and progression of breast cancer.

1.8.1 Statins

Statins are cholesterol lowering therapies, that inhibit the rate limiting enzyme of cholesterol synthesis, HMGCR, which converts HMG-CoA to mevalonate (Nelson *et al.*, 2013b). In humans, the use of cholesterol-lowering treatments, especially, statins have been shown to have a protective effect against breast tumour deaths and recurrence in older women (Anothaisintawee *et al.*, 2016; Borgquist, Giobbie-Hurder, Thomas P. Ahern, *et al.*, 2017). A recent meta-analysis demonstrated that breast tumour patients who regularly use statins, particularly lipophilic statins, display significant improved recurrent-free, overall and cancer- specific survival (Manthravadi, Shrestha and Madhusudhana, 2016; B. Liu *et al.*, 2017). Furthermore, a retrospective cohort study was done in 20,559 Swedish breast cancer patients who use statin, was linked with improved progression-free survival (with confidence intervals of 95% CI 0.63–0.95 and P = 0.014)

and lower risk of breast cancer mortality (95% CI 0.75–0.93, P = 0.001) in patients with inflammatory breast cancer (Borgquist, Giobbie-Hurder, Thomas P Ahern, *et al.*, 2017). Furthermore, the use of cholesterol lowering drugs, such as, fluvastatin was found to decrease breast cancer metastasis in a murine breast cancer metastasis model (Vintonenko *et al.*, 2012). Importantly, atorvastatin was also reported to reduce serum levels of 27OHC and CYP27A1 expression in patients who had been diagnosed with breast cancer (Kimbung *et al.*, 2017a), and thus, suggested this as one mechanism by which statins could reduce breast cancer progression. Another is the ability of the cholesterol lowering drug, simvastatin to increase expression of the PTEN gene, the anti-proliferative tumor suppressor gene, which reduces the phosphorylation of the Akt signalling pathway, eventuating in reduced cancer cell survival and growth in the MDA-MB-231 xenograft breast tumor model (Ghosh-Choudhury *et al.*, 2010). The same results were found *in vitro,* the antiproliferative effects and cytotoxic effects of statins in breast tumour cells by elevating autophagy, cell cycle arrest and apoptosis(Brewer *et al.*, 2013; Afzali, Vatankhah and Ostad, 2016)(Brewer *et al.*, 2013).

1.8.2 Plant phytosterols

Phytosterols are plant sterols, which can reduce intestinal cholesterol leading to lower serum cholesterol levels of LDL-C, and have anticancer effects which may be explained by preventing oxidative damage (Llaverias *et al.*, 2013). Several studies *in vivo* have tested the role of phytosterols on breast cancer progression. In mice injected with breast cancer cells MDA-MB-231, supplemented with 2 % phytosterols, it was observed that phytosterols reduced tumour size and metastasis (Miettinen *et al.*, 1995). Furthermore,

reduced tumour size was also reported in female mice received carcinoma MCF-7 cells injection and supplemented with β-sitosterol, the most widespread phytosterol (Awad, Williams and Fink, 2001). The ability of phytosterols to prevent lipoprotein oxidation was suggested as a potential mechanism to explain a reduction in both the progression of tumour burden and mammary hyperplastic lesions in PyMT mice fed a high fat, high-cholesterol diet (Llaverias *et al.*, 2013). The results from different studies confirmed that phytosterols affect the tumour by inhibiting tumour metastasis, slowing cell cycle progression, altering signal transduction, inducing apoptosis and inhibiting angiogenesis (Bradford and Awad, 2007; Blanco-Vaca, Cedó and Julve, 2018).

1.9 Hypothesis and aims

1.9.1 Hypothesis:

Cholesterol and 27OHC promote breast cancer cell growth, migration and invasion and this is associated with modulation of the IGF system.

1.9.2 Study aims and objectives:

- To confirm the effect of LDL on cell proliferation, migration, invasion, and resistance to chemotherapy in breast cancer epithelial cell lines.
- To assess the effect of 27OHC on cell proliferation, migration and invasion in breast cancer epithelial cell lines.
- To investigate the involvement of the ERβ in the role of 27OHC on breast cancer cell migration/invasion, and its regulation of IGF and EGF receptors.
- To delineate the involvement of the IGFs/IGF-IR in the effects of cholesterol on breast cancer cell growth and invasion.

Chapter 2.

Material and methods

2.1 Cell culture techniques

2.1.1. Cell lines

Human breast cancer cell lines MCF-7, T47D, MDA-MB-231 and Hs578T were purchased from ATCC (American Type Culture Collection) (Teddington, Middlesex, UK).

MCF-10A cell line: this a non-tumorigenic epithelial cell line that was derived from fibrocystic mammary gland of a 37 year old Caucasian female.

MCF-7 cell line: This cancerous breast epithelial cell line was derived in 1970 from the pleural effusion from a metastatic site of a 54 year old Caucasian female.

T47D cell line: This cancerous breast epithelial cell line was derived from the pleural effusion from a metastatic site of a ductal carcinoma of a 69 year old Caucasian female.

MDA-MB-231 cell line: This cancerous breast epithelial cell line was derived from the pleural effusion from a metastatic site of a carcinoma of a 51 year old Caucasian. It is a highly invasive, aggressive, and poorly differentiated TNBC.

Hs578T cell line: This cancerous breast epithelial cell line was derived from metastatic site of the pleural effusion from a metastatic site of an adenocarcinoma of a 74 year old Caucasian.

Table 2. 1: The receptor status of each breast cancer cell lines:

Cell Line	ERα	HER2	PR	ERβ
MCF-10A	-	-	-	+
MCF-7	++	-	+	+
T47D	+	-	+	+
MDA-MB-231	-	-	-	++
Hs578T	-	-	-	+

This table indicates receptor status of each cell line- ERα and -β, PR and HER2

2.1.2. Equipment

General sterile cell culture plastics were acquired from Greiner Bio-One (Gloucestershire, UK), pasteur pipettes from Fisher Scientific (Loughborough, UK), serological pipettes from Corning Incorporated (Amsterdam, Netherlands), syringes from Terumo (Leuven, Belgium), and 0.2μM filters from Appleton Woods (Birmingham, UK). The haemocytometer was from Hirschmann (8100104) and cryogenic vials were purchased from NUNC (Roskilde, Denmark). The hoods were obtained from BIOMAT (Class II, Medical Air Technology, Manchester, UK), incubators were from SANYO (MCO-18AIC), and the centrifuge was from MSE (Centaur2).

2.1.3. Stock solutions

- **Growth Media (GM):** MCF-7, T47D, MDA-MB-231 and HS578T cell lines were seeded in Dulbecco's modified Eagles Medium (DMEM, BioWhittaker, Verviers, Belgium) enriched with 10% fetal bovine serum (FBS, Gibco, Paisley, UK).
- **Serum Free Media (SFM):** DMEM was supplemented with 50 μg/ml streptomycin (Celltech Pharmaceuticals, Slough, UK), 5% L-glutamine(LG), 50 IU/ml penicillin (Britannia Pharmaceuticals, Redhill, UK), 10mg/L apo-transferrin (Sigma Aldrich), 1.2g/L sodium bicarbonate (Sigma Aldrich) and 0.2% bovine serum albumin (BSA) (Sigma Aldrich).

- **Phosphate Buffered Saline (PBS)-**PBS was prepared by dissolving one tablet (Oxoid Ltd., Basingstoke, UK) per 200ml of deionized H2O; the pH was adjusted to 7.4 and the solution was autoclaved and stored at room temperature.
- **Paraformaldehyde in 4% PBS (PFA),** (Alfa Aesar)- It is fixation solution for cells and is a ready-to-use solution. It is stored at 4°C.
- **Trypsin-EDTA (TE)-** (ethylenediaminetetraacetic acid) (TE)-10 x (Lonza); to make a 1 x solution, trypsin was diluted 1:10 with sterile PBS and stored at -20°C in 5ml aliquots.
- **Trypan Blue Dye (TB)-**TB stain (Sigma Aldrich) was used for assessing the viability and number of the cells: TB was diluted from 0.4% to 0.165% in PBS and mixed with a cell suspension at a 1:1 ratio.
- **27- hydroxycholesterol (27OHC)-** Santa Cruz Biotechnology (Santa Cruz, CA, USA) the stock solution 1000 µM was prepared in 100% ethanol and stored at -80°C. The stock solution was dissolved in growth media to give a range of concentrations from 0.1-1 µM.
- **Erteberel (LY500307), Cat# B1518-5m (Sigma Aldrich) -** it has selective binding affinity for estrogen receptor β (Erβ). The stock solution (10mM) was prepared in 1.77 mL DMSO and stored at -20°C. The stock solution was dissolved in growth media to give a range of concentrations from 0-10µM.
- **Lipoprotein, low density from human plasma (LDL)-** was bought from Thermo Fisher Scientific (Waltham, MA, USA). The stock solution was dissolved in growth media to give a range of concentrations from 0-100µg/ml.

- **4-[2–phenyl-5,7–bis (trifluoromethyl) pyrazolo [1,5-a]-pyrimidin-3-yl] phenol (PHTPP)** (SML1355-5MG), (Star lab), it is a selective ERβ antagonist. The stock solution was prepared in DMSO: 5 mg/mL, and stored at -20°C. The stock solution was dissolved in growth media to give a range of concentrations from 0-10μM.

2.1.4. Resuscitation of cells from liquid nitrogen

Cells stored in cryovials were taken from liquid nitrogen and placed briefly in a water bath at 37^0C to defrost. GM (1ml) was added to the tube and the cells were placed into a universal tube containing 9ml of GM. The cells were spun gently at 1,000 rpm for 3-5 minutes. The supernatant was removed by suction and the cell pellet was re-suspended in 5ml of GM using a syringe and 21G needle (Terumo).

2.1.5. Cell counting for seeding experiments

50μl of cell suspension were added in a 1:1 ratio with TB;0.165%). 50 μl of this mixture were added to the top and bottom counting chambers of a haemocytometer. The cells included within the chambers were counted using a light microscope. The non-viable cells stained blue whereas the viable cells were colourless. During cell death, the cell membrane becomes permeable that enables the TB dye to enter the cell and stain it blue. This does not occur in viable cells. The cells in both chambers were counted and multiplied by 1 x 10^4 to calculate the number of cells per ml.

2.1.6. Cell passaging

Cells were trypsinised when they reached ~80-90 % confluence, (1ml/T75 flask, 5ml/T175 flask). Growth media was removed, and cells were washed with PBS which was then aspirated and discarded. The cells were incubated with 1ml of 0.25% TE for 5 minutes at

37°C, 5% CO2. The flask was firmly tapped to detach the cells from the bottom of the flask. To deactivate the TE, 10ml of GM were added to the flask. The cell suspension was transferred to a universal container and centrifuged at 1200 rpm for 3-5 minutes, and then the supernatant was discarded. GM (5ml) was added to the pellet and the cells were re-suspended using a needle and syringe. 50µl from the cell suspension was added to an eppendorf for cell counting (section 2.1.5) to estimate the number of cells in the 5ml suspension prior to re-seeding. Depending on the cell type (Table 1), the cells were seeded with varying densities depending upon the vessel being used: T25, T75, or T175 flasks, as described in table 1.1. Media was changed every 2-3 days.

Table 2.2: Cell densities for T25, T75 and T175 flasks for each cell line.

Flask	Cell line	Cell density (x 10^6)	Company
T25	MCF-7/MDA-MB231/T47D/HS578T	0.2-0.3	Greiner BioOne
T75	MCF-7 /T47D	0.5	Greiner BioOne
	MDA-MB231/HS578T	0.8	Greiner BioOne
T175	MDA-MB231/HS578T	1.0	Greiner BioOne

2.1.7. Cell freezing

A cell suspension was prepared with $3x10^6$ cells for MCF-7 and MDA-MB-231 cells and $6x10^6$ cells for the HS578T cells in 500µl of growth media, and added to a cryogenic vial (Starlab, E3110-6122): a freezing mixture (500 µl) containing100µl of 10% dimethylsulfoxide (DMSO, Fisher Scientific, D/4121/PB08), 350µl of 85% GM, and 50µl of 5% FBS were added dropwise after it had cooled. Cryogenic vials were left in an insulating freezing container that would reduce the temperature in a controlled manner and kept at -80^0C overnight. After 24 hours the cryogenic vials were transferred to the liquid nitrogen tank and their position recorded in a log-book.

2.2. Experiment seeding densities

Seeding density for each experiment varied depending on the seeding plate/flask capacity and on the difference in cell size for each cell line. The following table 1.2 shows the different densities used for each experiment:

Table 2.3: Cell densities for specific assays

Experiment	Cell type	Seeding container	Seeding density
High Glucose experiments	MCF-7 & T47D	T75 flask	4.5 x106
Proliferation Assay Crystal Violet(CV)	MCF-7 & T47D	96 well plate	10000
Proliferation Assay (CV)	HS578T & MDA-MB-231	96 well plate	5000-8000
Any other experiment	MCF-7, T47D & H578T	6 well plate	0.1 x106

2.3. Cell treatments & dosing protocol

Cells were seeded in GM for 24 hours, before being transferred to SFM for a further 24 hours, after which the cells were dosed. To assess the effects of 27OHC and LDL, cells were treated with 0-0.1μM, and 0-100μg/ml respectively for 48 hours. While the concentration of the ERβ agonist and antagonist ranged from 0-10μM for 24 hours. Following treatments, cells were harvested using trypsin, then cells were centrifuged, and cell pellets were washed with PBS. Following cells were applied to different protocols such as the protein extraction, cell counting and sometimes reseeded for migration assays

2.4. Cell counting for experiments

2.4.1. Counting and viability assay using the Muse cell analyzer (Merck Millipore)

The media from each well was removed and added to a universal tube. Trypsin-EDTA (300μl) were added to each well and the plates were placed in the incubator at 37^0C for 5 minutes. The collected media was spun down at 10,000 rpm for 3 minutes to collect dead cells in a pellet and the resulting cell-free supernatant was collected in 7ml bijous and frozen for later use. After the 5-minute incubation, 1ml of GM was added to each well of the plates to neutralize the trypsin. The cell suspension was added to the universal tubes containing the pellet of dead cells, then spun down at 10,000 rpm for 5 minutes to form a pellet of live and dead cells. The pellet was re-suspended in 1ml of GM and 20 μl in triplicate of cell suspension were mix with 180 μl of Muse cell count and viability solution (1:20) to stain cell samples. After a 5-minute incubation, sample was loaded onto the Muse cell analyzer to read the results. For each condition, one sample at a time both viable and dead cells were counted and reported.

2.4.2. Crystal Violet (CV) assay for determining viability of cultured cells with CV staining

The cells in 200µl GM were seeded into 96 well plates with 3-10 replicates per each condition for 24 hours. Then, the GM was removed and added 200µl SFM for a further 24 hours, and the cells were dosed with specific treatment such as, LDL, 27OHC. After incubation, the plate was fixed by applying 100µl 4% PFA for 20 min. The fixative was removed and added PBS. Then, the cells were washed two times with PBS followed by the addition of 100µl CV solution (0.05% in PBS) and then incubated for 30min. After that, the plate was washed three times with 200µl/well PBS. The last step was replaced the PBS with 100µl/well of 1% SDS/PBS. To solubilize the CV the plate was placed on a rocker for 1 hour and read using a plate reader (FLUOstar Optima, BMG LABTECH).

2.5. Transfection with siRNA

Table 2.4: Reagents:

ERα siRNA (20nM) **ERβ siRNA (20nM)**	Dharmacon
CYP27A1 siRNA (20nM),	Thermo Fisher Scientific
AllStars Neg. Control siRNA (20nM)	Qiagen
siRNA Transfection Reagent saint red	Synvolux
Growth media	DMEM 5mM glucose

Lyophilised siRNA was resuspended with an appropriate amount of siRNA suspension buffer to make a 20µM stock solution, which was aliquoted and stored at -20°C. The

transfection master-mix depended on its required concentration: for example, a 20nM solution for ERα siRNA was prepared, as illustrated in table 2.4, followed by 20 minutes incubation period at the room temperature. The stock cells were trypsinised when 70-80% confluent, counted and then seeded into 6-well plates ($0.1X10^6$/cells in 800µl /well of GM). 200 µl of the transfection master-mix were added gently drop-wise. The cells were then incubated for 24 hours before changing the media to SFM for a further 24 hours at 37°C in 5% CO2. The transfection efficiency was assessed by western immunoblotting.

Table 2.5 : Reagents was used for preparing the transfection master-mix

Reagent	**Volume per well- ERα siRNA**	**Volume per well- ERβ siRNA**	**Volume per well- CYP27A1 siRNA**
Buffer	20ul	20ul	20ul
siRNA transfection reagent Saint-RED	9ul	9ul	9ul
siRNA	ERα siRNA (20nM)	ERβ siRNA (40nM)	CYP27A1 siRNA (40nM),
Growth media (DMEM)	Top up to 200ul total volume per well	Top up to 200ul total volume per well	Top up to 200ul total volume per well

2.6 Development of stable transfection of ERβ using shRNA

Transduction with lentiviral vectors is defined as a highly efficient method capable of transducing cells to adjust expression of target genes (Blömer *et al.*, 1997). Short hairpin RNA (shRNA) applied to target the human ERβ gene (ESR2) (ERβ shRNA (h) Lentiviral Particles: (sc-35325-V) were obtained from Santa Cruz Biotechnology, Inc. ERβ shRNA (h) lentiviral particles is a combine of concentrated, transduction prepared viral particles including three target-specific constructs that encode (19–25 nucleotide sequences in length, plus hairpin) shRNA designed to silence gene expression. As a negative control, control shRNA lentiviral Particles-A (sc-108080) that encode a mix-up shRNA sequence were used in this model.

MDA-MB-231 (high metastatic potential, ERβ- positive), were harvested by trypsinization and seeded into 6-well plates. Infections of MDA-MB-231 cells were performed with two volumes (15-20µl) of shRNA target-specific human ERβ or non-targeting shRNA control. Polybrene solution (5µg/mL) (sc-134220; Santa Cruz Biotechnology, Inc) were used to optimise transfection efficiency, following the manufacturers protocol.

After 24h incubation time, the medium was changed to complete medium without polybrene. After a further 24h, the transfected cells were split 1:3 and incubated for 48h in complete medium.

Puromycin kill curve experiments were performed with 0.2-1µg/mL to assess the appropriate concentration of puromycin to kill 100% of untransfected cells in one week and allow the survival of the remaining transfected puromycin treated cells that are stably transfected.

For the development of stable clones that express shRNA, puromycin dihydrochloride selection (sc-108,071; Santa Cruz Biotechnology, Inc) 0.8µg/mL was supplemented in the culture media, changing it with fresh puromycin-containing media once every 3–4 days. Transfection efficiency was assessed by western blotting and qPCR.

2.7. Trans-well migration/invasion assay using crystal violet staining

Cells were seeded and following treatment were trypsinised and resuspended in SFM in preparation for the migration/invasion assay. Trans-well inserts containing polycarbonate filters (8 µm pores-transwell) (Millipore) were used in 24-well plates. To detect invasion, the inserts were coated with 10µg/ml collagen-I for 24 hours before cell seeding at room temperature. Cells (5×10^5) / per well were seeded in the upper chamber of the inserts in triplicate in SFM-DMEM and incubated at 37^{o}C. DMEM containing 5% FBS was used in the lower chambers to act as a chemoattractant for the cells. Cells migrated against the chemoattractant medium in the lower chambers for 24 h (MCF-7) or 6 h (MDA-MB-231). The cells that had passed through the membrane to the bottom side were fixed using 300µl of 4% paraformaldehyde for 15 minutes. The membrane was washed with PBS permeabilized with triton X, stained with crystal violet, incubated for 20 minutes and washed with PBS. Cotton buds were used to remove any non-migrated cells from the top-side of the membrane. The stain was then dissolved in 1% SDS on a plate shaker for one hour. The plate was read the optical density values utilizing an iMark plate reader (BioRad).

The traditional method for assessing cell migration/invasion is counting the cells that have migrated/invaded through the membrane, but more recently dye extraction methods have been employed.

The trans-well migration assay was performed using MCF-7 cells to examine changes in cell migration, after which the migration rate was assessed using both techniques for comparison. Figure 2.2. confirms that the techniques are comparable and overall dye extraction was quicker, cheaper and more reproducible than counting.

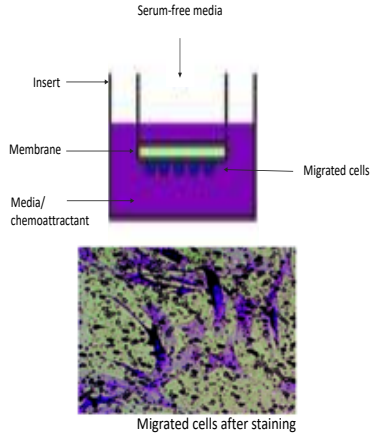


Figure 2. 1: Trans-well migration assay using crystal violet staining.

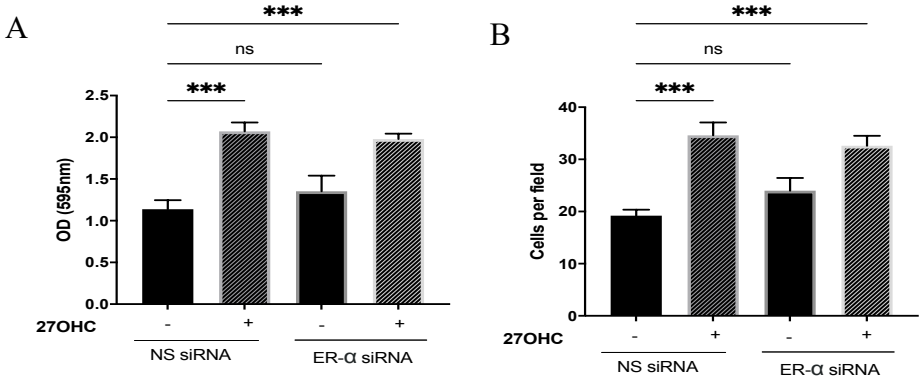


Figure 2. 2: Optimization of migration and invasion in MCF-7 cells using a trans-well migration assay and assessing using two techniques: counting invading/migrating cells and a dye extraction method

Cells were treated with LDL (80µg/ml) for 48 hours. A trans-well assay was used to detect cell migration and invasion after 24hours. The migrated cells were stained with crystal violet and quantified by A) analysis of extracted stain using a microplate reader at an OD at 595 nm and B) by count the stained, migrated cells. Data are representative of the mean ±SEM (n=3). P-values were determined by using GraphPad.

2.8. Western immunoblotting (WB)

2.8.1. Preparation of cells lysates

The preparation of the lysis buffer is described in table 2.6. 10µl/ml of the protease inhibitor cocktail and 10µl/ml of the phosphatase inhibitor cocktail were added to the lysis before it was used. An appropriate volume of lysis buffer (depending upon the number of cells/sizes of the pellet) was added to each cell pellet. It was, re-suspended, centrifuged for 5 minutes at 15,000 x g at 4°C. The supernatant (containing the proteins) was transferred to a fresh tube, left at 4^0C for 20 minutes and then stored at -20^{o}C.

Table 2. 6: Ingredients of lysis buffer and protein standards concentration range

Lysis Buffer	**Quantity**
Tris HCL (Sigma)	1.576g/L
Sodium Chloride (Sigma)	2.92g/L
EDTA (Sigma)	1.86g/L
Sodium Pyrophosphate (Sigma)	6.66g/L
Sodium Fluoride (Sigma)	2.1g/L
Sodium Orthovanadate (Sigma)	100µM- (10mls of 100x solution)
Triton X-100 (Sigma)	1%- 1(10mls/L)

	Standard concentration	**BSA (µl)**	**Lysis buffer**
A	2000 μ g/ml	300 μ l of stock	0
B	1500 μ g/ml	375 μ l of stock	125
C	1000 μ g/ml	325 μ l of stock	325
D	750 μ g/ml	175 μ l of stock B	175
E	500 μ g/ml	325 μ l of stock C	325
F	250µg/ml	325 μ l of stock E	325
G	125 μ g/ml	325 μ l of stock F	325
H	25 μ g/ml	100 μ l of stock G	400

2.8.2. Protein quantification

To assess the total protein in the cell lysates, protein concentration was observed using the Pierce bicinchoninic acid (BCA) protein assay kit, containing BCA reagent A and B, (Pierce Biotechnology, Cheshire, UK) according to the manufacturer's instructions. Protein standards, stock standard of 2mg/mL, containing BSA in 0.9% saline and 0.05% sodium azide in lysis buffer, was used to make a variety of concentrations from 0.025-2.0mg/ml, as described in table 2.6. Into a 96 well plate, 5µl of the protein standards with 5µl of each sample were placed in duplicate. Then, reagents A and B from the protein assay kit, were combined in a ratio of 50:1 and 200µL were placed to each of the targeted wells. Then the plate was left for 30 minutes. After the incubation period the plate was read utilizing a plate reader at a wavelength of 590nm using the Bio-Rad software package. The concentration of each sample was calculated (in mg/ml) by reading from the standard curve.

2.8.3. Sample preparation and gel electrophoresis

Samples were prepared by diluting to a correct concentration according to the measured protein concentration in PBS. Protein samples were resuspended in 2X Laemmli Sample Buffer, containing glycerol to increase the density of sample, anionic detergent (SDS), and bromophenol blue dye to allow visualization, and denatured by boiling for five minutes at 95°C.

Table 2. 7: Western blotting reagents:

Reagents	Description
30% Acrylamide/ BIS (N,N'-methylene bis acrylamide). (Severn Biotech)	4°C storage degree.
1.5 M Tris Base (Sigma), (18.16g) pH 8.9	The reagents made up in 70mls of distilled water (dH2O) with pH adjusted and the solutions were added to 100mls and stored at 4°C.
1.1 M Tris HCL (Sigma) (17.33g) pH 6.8	
10% Sodium dodecyl sulphate (SDS), pH 7.2 (Sigma)	50g Electrophoresis grade SDS was dissolved in 400mls dH2O and heated to 68°C with pH adjusted before the solution was added to 500mls. The solution was filter sterilized and stored at room temperature
10% Ammonium persulphate (APS) (Sigma)	1g made up in 10mls of dH2O and stored at 4°C.
TEMED (N,N,N,N-tetramethylethylenediamine) (Sigma)	Stored at room temperature.
10x Tris-buffered saline TWEEN 20 [TBS-T]	50 mM Tris base pH 7.5, 5ml 0.1% Tween-20 and 150 mM NaCl, dissolved in 500ml dH2O. The solution was calibrated with HCl and NaOH for a pH value of 7.6.
Blocking solution:	5% BSA dissolved in 1x TBS-T.

Table 2. 8 : Chemicals and buffer for western blotting.

SDS polyacrylamide gel electrophoresis	**10% resolving gel (50–200kDa)**	**5%stacking gel:**
Distilled water	9.6ml	9.6ml
40% acrylamide	37.5:1	37.5:1
Triss buffer	1.5 M pH 8.8	0.5 M pH 6.8
10% SDS	0.1%	0.1%
10% APS	0.1%	0.1%
0.0006% TEMED	0.06%	0.1%
1X Running buffer	25 mM Tris Base tris-HCl pH 6.8 0.1% SDS 192 mM Glycine	
10X Transfer buffer	25 mM Tris-HCl pH 8.3 192 mM glycine 20% methanol	

The resolving and stacking gels were prepared as described in Table 2.7. The resolving solution portions are applied between glass plates (1.5mm apart|) and enabled to polymerise under a layer of butanol, which makes sure a smooth interface and speeding up the polymerisation process. After the gel has set, the gel was washed with dH_2O before adding the stacking gel and inserting a comb(10-well). Once the stacking gel has polymerised, the comb was pulled out and the gels was transferred to the running tank. The electrode chambers and tank were topped up with running buffer before loading with the samples. Samples were allocated in SDS polyacrylamide gel electrophoresis (SDS-PAGE), then the gel was run at (125–200V) according to the number of gels in the tank, for 45–90 minutes depending upon the separation required and protein size, and filled with 1x running buffer, as indicated in table 2.8

2.8.4. Wet transfer western blot

The separated proteins were transferred to a western blot membrane (nitrocellulose membrane, PVDF (polyvinylidene difluoride). The membrane was pre-soaked for 10 minutes in 1X transfer buffer along with the gel, sponge and thick paper. A transfer sandwich was made of sponge – thick paper – gel – membrane – thick paper – sponge, and placed in a transfer tank, loaded with 1x transfer buffer, and run by limited volts (90V) for 90 minutes at 4°C. After the proteins had transferred from the gel to the membrane, the membrane was washed in tris buffered saline tween (TBST) for 3 x 5minute washes before being blocked for 1 hour at room temperature with blocking solution (5% non-fat dried milk or 3-5% BSA in TBST) with gentle agitation. The primary antibody solution was prepared with blocking solution containing 5% non-fat dried milk or 3-5% BSA in TBST (according to the manufacturer's specification) as indicated in Table 2.9 and the membranes were incubated for 18 hours at 4°C.

Membranes were then washed with TBST for 3 x 10 minute at room temperature and incubated with secondary antibody diluted (1:3,000 for anti-rabbit and 1:1,000 for anti-mouse) at room temperature in fresh blocking solution for 1 hour, then the membrane was washed three times with TBST for 5 minutes each at room temperature.

The membrane was incubated for 5 minutes with Clarity Luminata Forte ECL substrate (Bio-Rad) or Femto (Pierce, Rockford, IL) before being visualized by using Bio-Rad ChemiDoc (system and Image lab software). Image J 1.46r software (National Institutes of Health, Bethesda, MD) was used to quantified Western blots after scanning.

Table 2.9 : Western blotting antibodies

Antibody	Company	Concentrations
Anti-Liver-X-Receptor (LXR-β)	Cell Signalling Technology	1:1000
Anti-Insulin Growth Factor I -β (IGF-IRβ)	Cell Signalling Technology	1:1000
Anti-Phospho-IGF-I Receptor β (pIGF-IRβ)	Thermo Fisher	1:1000
β 1 integrin	Merck Millipore	1:1000
Anti-Estrogen receptor β (ERβ)	Merck Millipore	1:2000
Anti-Estrogen receptor β (ERβ)	ABCAM	1;1000
Anti-Estrogen receptor β (ERβ)	Thermo Fisher	1:1000
Anti-Estrogen receptor α (ERα)	Santa Cruz Biotechnology	1:1000
Anti-Fibronectin	BD Transduction	1:1000
Anti-E-cadherin	Cell Signalling Technology	1:1000
Anti-Vimentin	BD Transduction	1:500
Anti-CYP27A1	ABCAM	1:1000
Anti-MAPK	Thermo fisher	1:1000
Anti-Phospho-MAPK	Thermo fisher	1:1000
Anti-AKT	Cole-Parmer Scientific experts	1:1000
Anti-Phospho-AKT	Star Lab	1:1000
Anti-Low-density Lipoprotein-Receptor (LDL-R)	Merck Millipore	1:1000
Anti-α Tubulin	Sigma	1:5000
Anti-β Actin	Sigma	1:10,000
Anti-GAPDH	Chemicon	1:5000

2.9. Quantitative real-time polymerase chain reaction (qRT-PCR)

2.9.1. RNA extraction and quantification

- **Solutions and reagents**

- Isopropanol
- Ribozol extraction reagent
- 99.8+% Chloroform
- 70% ethanol, prepared by mixing 35ml 99.8% ethanol and 15ml molecular biology grade, nuclease-free water
- Trizol
- Molecular biology grade, Nuclease free water

- **Materials and equipment**

- Refrigerated centrifuge (PrismR, Labnet International)
- Greiner 1.5ml Microcentrifuge Tubes (Greiner Bio-one, 616201)
- Nanophotometer (Implen, Munich, Germany)
- The autoclaved tubes.

- **Procedure**

Cells were lysed by adding 1ml of Trizol directly to the culture dish for 5 minutes at room temperature. Cell lysates were transferred to sterile 1.5ml microcentrifuge Eppendorf tubes and incubated at room temperature for 5-7 minutes to dissociate nucleoprotein complexes. Chloroform was added (0.2ml per 1ml of the cell lysate). Each eppendorf was shaken for 15-seconds and was incubated for 10-15 minutes at room temperature. These samples were centrifuged at 4^0C 12000xg (PrismR, Labnet International) for 15 minutes, to separate the sample into 3 layers of an aqueous layer containing RNA (clear top), interphase layer

(middle white layer), and phenol/chloroform/DNA/protein mixture (pink bottom layer). The aqueous phase containing RNA was transferred to a 1.5ml eppendorf tube and incubated with 0.5ml isopropanol (Fisher Scientific, 10284200) at room temperature for 10 minutes. Samples were centrifuged at 12000xg for 10 minutes at 4ºC. The supernatants were carefully discarded, and the RNA pellet was washed with 1ml of 70% ethanol (Fisher Scientific, 10437341). The RNA pellet was centrifuged at 8600x(rpm) for five minutes, the ethanol was discarded, and the pellet left to air dry. The RNA pellet was dissolved in 10-12µl of RNase-free water and vortexed to dissolve the RNA. The amount of RNA extract was quantified using a nanophotometer (Implen, Munich, Germany). The sample purity was read at the ratio of 260 to 280 with a ratio of between of 1.8-2.0.

2.9.2. RNA purification

Deoxyribonuclease I (DNase I) was added to extracted RNA, to eliminate any genomic DNA contamination, using a DNase I Kit (Thermo Fisher Scientific, 18047019) according to the manufacturer's instructions. Briefly, the sample was diluted with 8µl of DNase-free water and mixed with 2µl of the master-mix, which was prepared by adding 1µl of DNase (volume/reaction) and 1µl of 10x reaction buffer, and incubated at room temperature for 15 minutes, followed by the addition of 1µl DNase stop solution. After incubation of the RNA samples for 10 minutes at 70ºC, the samples were placed on ice in preparation for the reverse transcription step.

2.9.3. Reverse transcription

- **Solutions and reagents**

- Molecular biology grade, nuclease free water (Thermo Scientific, SH30538.01)

- High-capacity RNA-to-cDNA kit (Applied Biosystems, P/N 4387406). The kit contains;
    - 2X RT Buffer mix (includes dNTPs random octamers and oligo dT-16)
    - 20X RT Enzyme mix (MuLV reverse transcriptase and RNase inhibitor protein)

- **Materials and equipment**

- Refrigerated centrifuge (PrismR, Labnet International)
- Thermal cycler PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA)
- PCR tubes (Alpha laboratories)
- Vortex mixer (Vortex genie, Scientific Industries Inc.)

- **Procedure**

The cDNA was created by using the high-capacity RNA-to-cDNA kit reagents prepared as listed in the table 2.10. (Applied Biosystems, P/N 4387406).

Table 2.10: Preparation of the master-mix used for reverse transcription, as manufacture instruction.

Component	Volume per reaction (µl)
2X reverse transcriptase (RT) Buffer (includes dNTPs, random octamers, and Oligo dT-16)	10µl
20X RT Enzyme (MuLV and RNase inhibitor protein)	1µl

1µl of RNA sample was diluted with 9µl DNase/RNase-free water and mixed with 11µl of the master-mix generated on ice using the ingredients listed in Table 2.10. The tubes were briefly centrifuged. The samples were added to the PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA), with a negative control (RNA treated as described but with no

addition of the reverse transcriptase buffer). The program was set to incubate RNA samples at 37°C for 60 minutes, heated up to 95°C and cooled down to 4°C, this allowed the reaction to stop. The samples were stored at -20°C for further use.

2.9.4. Quantitative PCR (qPCR)

- **Solutions and Reagents**

- SYBR Green Jumpstart™ Taq Readymix™ (Sigma, S4438) containing 20 mM tris-HCl, pH 8.3, 7 mM $MgCl_2$, 100 mM KCl, 0.4 mM each dNTP (dATP, dCTP, dGTP, TTP), stabilizers, 0.05 unit/ml taq DNA Polymerase, JumpStart Taq antibody and SYBR Green I.
- 100X Internal Reference Dye (Sigma, R4526)
- ERβ primer pairs (Sigma)

  Forward: AAGTTGGCCGACAAGGAGTT

  Reverse: ACAGGCTGAGCTCCACAAAG
- GAPDH primer pairs (Sigma)

  Forward: CAATGACCCCTTCATTGACC

  Reverse: GATCTCGCTCCTGGAAGATG
- Molecular biology grade, Nuclease free water (Thermo Scientific, SH30538.01)

- **Materials and equipment**

- StepOne Plus Real Time, PCR System (Applied Biosystems, Foster city, CA)
- 96-well PCR plates (Sarstedt, 95.1994)
- Plate centrifuge (MPS 1000 Mini PCR Plate Spinner)
- PCR tubes (Alpha laboratories, LW2340)
- Optically clear sealing tape (Sarstedt, 95.1994)

- **Procedure**

qPCR based on the detection of PCR products produced by reactions with a fluorescent molecule that reports a proportional elevation in the fluorescent signal with elevation in the amount of DNA. Using SYBR green, the master mixes were prepared as described in table 2.11, for each sample and a negative control. The mix was prepared in PCR tubes, by adding cDNA to the master-mix. The samples were vortexed and placed into a 96-well plate in duplicate. The negative control was prepared by adding water and cDNA (without the reverse transcriptase enzyme) in duplicate, to check for possible contamination.

Table 2. 11: Preparation of qPCR master mix.

Component	Concentration per reaction
2X SYBR green	1X
100X Internal Reference Dye	0.1X
Forward primer (10 µM)	0.2-1µM
Reverse primer (10 µM)	0.2-1µM
PCR water	As required

The 96-well plates were sealed and centrifuged for 10 seconds on a plate spinner to ensure the mixtures were at the bottom of each well. The plate was placed on the ABI StepOne Plus Real Time PCR System), and the program was set for the thermal cycling parameters as described in Table 2.12.

Table 2. 12: qPCR thermal cycling parameters used in the protocol.

	Temperature	Duration	
Initial denaturation	95°C	120 minutes	40 cycles
Denaturation	95°C	30 seconds	
Annealing Extension Recording fluorescence	60°C	60 seconds	

The target gene was normalized to a reference gene, GAPDH, and expressed as relative RNA levels against the control gene, calculated by using the $2^{-\Delta Ct}$ method as previously described (Kondaveeti, Guttilla Reed and White, 2015).

2.10 Radioimmunoassay (RIA) for IGF-I

Table 2. 13: Reagents used for radioimmunoassay.

Reagent	Description
Assay buffer	Formed by 0.2g Protamine sulphate (Sigma – P4020), 0.03M sodium dihydrogen phosphate (BDH – 301324Q), (3mM) sodium azide (Sigma – S2002), (10mM) EDTA (Sigma – E5134), and (0.05%) TWEEN 20 (Sigma – P5927). Added to 1L dH2O, adjusted to pH 7.5 and stored at 4°C.
8M formic acid	Formed by 8M of neat formic acid added to 100ml of ddH2O, and 3–4 drops of TWEEN 20 were added to give approximately 0.05% TWEEN (stored at room temperature).
1M Tris base	Formed by adding 1M of Tris base to 100ml of ddH20, stored at room temperature.
Acetone	(BDH, Poole, UK)

An extraction procedure was carried out to release IGF-I from their endogenous binding proteins. 50µl of the sample and a control of normal human serum were aliquoted into 1.5ml eppendorfs. 25µl of the formic acid/TWEEN solution were added to each sample and vortexed. 175µl of acetone were added to each sample, by using an acetone primed pipette tip. The samples were immediately capped, vortexed and centrifuged, for 30 minutes at 4^{o}C at a rcf of 3000g. In a fresh eppendorf, 100µl of supernatant were placed and mixed with 100µl of tris base. The conditioned media did not need dilution, therefore, the standards were added with 50µl of acid/ tris base solution /acetone, with 50µl of the conditioned media to make sure that all conditions were maintained. Normal human serum was used as positive control, which was diluted 1:80 (in assay buffer).

Recombinant human IGF-I standards were prepared in assay buffer to produce a standard curve with the following dilutions for IGF-I: 0, 0.25, 0.5, 1.0, 2.5, 5, 10, 25 & 50ng/ml. Freshly made radioactive tracer with I^{-125} labelled IGF-I was prepared by adding 25ml of assay buffer to 50µl of the labelled tracer. Using a bench-top gamma counter, 50µl of the mix solution were placed into a test tube and the number of counts were measured. The number of counts should be between 10,000-15,000 cpm per assay tube.

IGF-I radioimmunoassay antiserum was a monoclonal antibody (Clone M23/ILG1-001, 5345-0304) diluted with assay buffer at a 1:2000 dilution.

RIA is very sensitive to residual binding proteins, therefore, an excess 200ng/ml of IGF-II peptide were added to the assay to removes any interference from them after the extraction procedure. The peptide will not change or affect the levels of the measured IGF-I because they would not be recognised by the very specific antibodies. Then, the tubes were labelled for total counts of the tracer (TC) in duplicates, non-specific binding (NSB), which demonstrated the level of iodinated peptide binding to the tube, usually less than 3% of TC, maximum binding (around 40% of TC), and each assay standard. Tubes were labelled for the samples and were set up in triplicate, as indicated in Table 2.14 for IGF-I.

Each tube was vortexed (except for TC tubes), covered and incubated at 4°C (in the fridge) for 24h. 50µl of anti-mouse SAC-CEL (IDS Ltd., Bolden, UK) were added to tubes (except for the TC tubes). The tubes were vortexed, and incubated for 30 minutes at 4°C. SAC-CEL is a solid-phase (covalent-coupled) secondary antibody coated cellulose suspension that pulls down the primary antibody after centrifugation and this step was performed with continuous stirring. Distilled water 1ml was added to tubes and centrifuged at 2000g at 4°C for 30 minutes (except the TC tubes). The IGF-I was then released from the SAC-CEL by

adding the acetic acid and the tubes then centrifuged again, as before. The generated supernatant was extracted and counted using the Gamma Counter. The concentration of IGF-I ligand was then calculated automatically according to the standard curve by a pre-set computer program.

Table 2. 14: IGF-I RIA reagent preparation

	Assay buffer (µl)	**Sample (µl)**	**Standards (µl)**	**Tracer (µl)**	**Antibody (µl)**	**IGF-II (µl) (excess)**
TC	-	-	-	50	-	-
NSB	400	-	-	50	-	50
Maximum binding	350	-	-	50	50	50
Standards	300	-	50	50	50	50
Samples	300	50	-	50	50	50

Chapter 3.

The effects of LDL on breast cancer cell proliferation, migration, invasion, and drug resistance.

3.1 Introduction

Obesity has been identified as a leading risk factor for breast tumour incidence in post-menopausal women and abnormal lipid accumulation and cholesterol may affect breast cancer progression and treatment (Bradley, 2018; F *et al.*, 2018). Cholesterol associates with lipoproteins, to be transferred to several parts in the body. Lipoproteins include low-density (LDL) and high-density lipoproteins (HDL) (Li *et al.*, 2018). Several studies have evaluated the role of lipid accumulation with breast cancer risk, and found increased levels of LDL, total cholesterol and triglyceride in breast cancer patients when compared with healthy women, with LDL cholesterol being significantly correlated with breast cancer progression (Rodrigues Dos Santos *et al.*, 2014; Kumar *et al.*, 2015).

Cholesterol is also an important component for the cell membrane as well as for cell proliferation and differentiation (Silvente-Poirot and Poirot, 2014). Moreover, cholesterol is a crucial factor of lipid rafts, which are sphingolipid enriched domains of the plasma membrane (Park *et al*., 2010). Lipid rafts contain different cell signalling molecules, such as EGFR, Ras, FAS/CD95, and Wnt that contribute to the regulation of cell proliferation, mobility and survival (Park *et al*., 2010). Cholesterol accumulation has been demonstrated in breast cancer patients with higher levels in breast tumours compared to normal tissue (Silvente-Poirot and Poirot, 2014). Also, cholesterol accumulation may activate the interaction of key signalling molecules in lipid rafts and thus enhance cell growth and survival by activating the cell surface markers such as the EGFR and the IGF-IR in cancer (Laurentiis, Donovan and Arcaro, 2007) (Lee Seong-Hee *et al.*, 2009). Additionally, a retrospective cohort study assessed the status of the serum lipids and lipoproteins in women breast cancer patients during chemotherapy at initial diagnosis. They found that

chemotherapy treatment caused significant changes in metabolic status, decreases in HDL-C and apolipoprotein (Apo A1) and increases in total cholesterol, triglycerides, LDL-C and Apo B (Li *et al*., 2018).

An epidemiologic study indicated that cholesterol and its metabolite contribute to the risk of breast cancer and overall survival (Dalenc *et al.*, 2017; Kimbung *et al.*, 2017a). (DuSell *et al.*, 2008). Oxysterols, such as 27OHC, oxygenated derivatives of cholesterol, are also thought to play a role in the development of breast cancer as indicated in experimental animal models (Oguro, 2019). A prospective study in breast cancer patients found a significant increase in 27OHC in the patients' blood serum in response to aromatase inhibitor (AI) treatment, and they suggested that 27OHC might be a marker of both poor prognosis and/or the efficacy of endocrine treatment (Dalenc *et al.*, 2017). Another recent prospective study indicated no correlation between circulating 27OHC level and breast cancer risk in postmenopausal-women and no association with ERα breast cancer status (Lu Da-Lin *et al.*, 2019). *In vitro*, whilst 27OHC alone increased cell proliferation through activation of the ER, administration of 27OHC decreased estradiol-induced proliferation (DuSell *et al.*, 2008).

Furthermore, it has been found that increased CYP27A1 levels, the enzyme that converts cholesterol to 27OHC, were observed in more aggressive mammary breast cancers (Nelson *et al.*, 2013b; Wu *et al.*, 2013). A study investigating the correlation between patients taking statins and risk of breast cancer are equally conflicting, with the meta analyses revealing that there is no correlation (Undela, Srikanth and Bansal, 2012). However, there is strong clinical data supporting a correlation between serum levels of total cholesterol and breast cancer survival and recurrence, with elevated total cholesterol correlating with increased

breast cancer recurrence (Bahl *et al*., 2005). A recent phase III, double-blind trial, found that cholesterol lowering treatment along with endocrine therapy was correlated with elevated recurrence-free survival time in 8010 invasive breast cancer postmenopausal women with hormone receptor positive (Borgquist, Giobbie-Hurder, Thomas P Ahern, *et al.*, 2017).

The anti-cancer drug doxorubicin has been widely used to treat different types of cancer including breast cancer, but its use is limited due to dose-dependent side effects (Doroshow, 1991). In cell line models, statins, influence the sensitivity of doxorubicin in ovarian cancer (Martirosyan *et al.*, 2010), breast cancer (T47D, MCF-7 and MDA-MB-231), and osteosarcoma (Werner, Sacher and Hohenegger, 2004; Fromigué, Hamidouche and Marie, 2008). These studies suggest that high cholesterol levels may interfere with chemotherapy resistance in breast cancer patients.

3.2 Aims

In this chapter, we aimed to assess the effect of LDL on cell proliferation, migration, invasion, and resistance to chemotherapy in breast cancer epithelial cell lines.

3.3 Specific aims

Aim 1: To examine the effect of LDL on cell proliferation, migration, and invasion in both ERα-positive and ERα-negative breast cancer epithelial cell lines.

Aim 2: To address the role of the CYP27A1 enzyme in the effects of LDL on cell proliferation and migration in both ERα-positive and ERα-negative cells, by silencing CYP27A1 using siRNA.

Aim 3: To address the effects of LDL and its metabolite 27OHC on the survival of breast cancer cell lines, in response to chemotherapy.

3.4. Methods

3.4.1. Cell culture:

The human breast cancer cell lines MCF7, T47D, Hs578T and MDA-MB-231 were obtained from ATCC (Teddington, Middlesex, UK). Each cell lines were grew as described previously in section 2.1.1.

3.4.2. Cell counting:

3.4.2.1. Optimising a crystal violet (CV) proliferation assay

The crystal violet assay is a simple and reliable method, because it directly stains the attached cells with crystal violet dye, which binds to DNA and proteins (Feoktistova, Geserick and Leverkus, 2016a). We performed some optimisation steps to ensure that the CV assay performed sensitively and reliably for future experiments. The CV assay was performed using MCF-7 cells to examine changes in cell proliferation at different seeding densities: 500, 1000, 2000, 4000, 8000, and 16000 cells per well for 24, 48, 72, and 96 hours, after which the proliferation rate for each density/time point was measured.

3.4.2.2 LDL treatment

For cell proliferation assays, cells were incubated in growth media DMEM for 24 hours, before being changed to serum-free media for a further 24 hours. The cells were then treated with different concentrations of LDL 0-100μg/ml. Crystal violet assay was used to determine the viability and proliferation of MCF-7 cells, as outlined in section (2.6).

3.4.3 Western blot

Western blot analysis was observed as described previously (2.10). In brief, protein cell lysates (30 μg), were allocated in SDS-PAGE gel, transferred to nitrocellulose membrane and immunoblotted with the targeted antibodies: anti-CYP27A1 (1:1000), GAPDH

(1:5,000; Millipore) and β-actin (1:10,000; Sigma-Aldrich). After that the membranes were incubated with particular secondary antibodies (anti Mouse or Rabbit) conjugated to peroxidase (Sigma), next proteins were detected by Clarity ECL substrate (BioRad) by using BioRad Chemidoc XRS + system and quantified by using Image J 1.46r software (BioRad).

3.4.4 Optimizing the CYP27A1 antibody

Antibody validation is critical to generate accurate, valid data. For experiments to investigate if the effects of LDL were through CYP27A1 (is the rate-limiting step in 27-hydroxycholesterol biosynthesis), we first validated the CYP27A1 antibody as shown in Figure 3.1. We used western blotting technique to assess the protein abundance of CYP27A1 in different breast cancer cell lines. The CYP27A1 antibody detected a single band of the expected size following the manufacture protocol (the molecular weight; 60kDa) in lysates from a panel of breast cancer cell lines (Fig. 3.1).

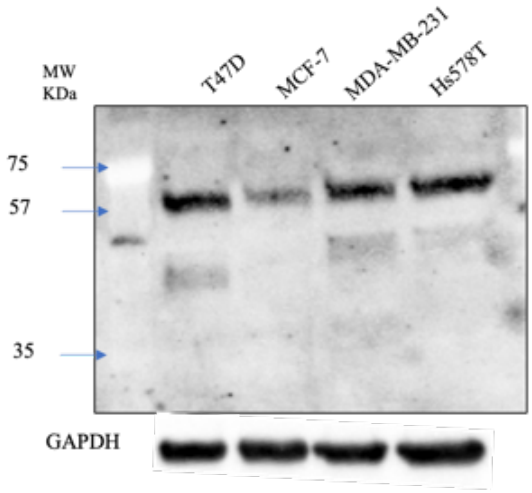


Figure 3. 1: Validation of the CYP27A1 antibody using a panel of breast cancer cells;

Representative images of western blotting for lysates from exponentially growing whole-cell lysates from 4 cancerous cell lines ERα-positive; MCF-7 & T47D and ERα-negative; MDA- MB-231 & Hs578T were used for these experiments: showing the band was detected at the correct molecular weight of 60KDa and showing the level of CYP27A1 in different breast cancer cells. GAPDH was used as loading control protein. Data representative of (n=1).

3.4.5 Targeted CYP27A1 gene knockdown, using siRNA knockdown

For experiments investigating the effect of LDL on the cell proliferation and migration of MCF-7 and MDA-MB-231 cells in the presence or absence of CYP27A1, the optimum concentration of siRNA for silencing the CYP27A1 (Dharmacon) or non-silencing (NS) (AllStars) was investigated. The cell number for this experiment was assessed using a Muse cell analyzer, as described in section 2.5.

CYP27A1 siRNA was optimised using a range of concentrations (20, 30 and 40nM) for ERα-positive cells (MCF-7) and (20 and 40nM) for ERα-negative cells (MDA-MB-231) and the level of knockdown of CYP27A1 protein expression was assessed by Western blot

analysis. The optimum concentration of siRNA for silencing the CYP27A1 was 40nM in MCF-7 (Fig. 3.2A) and MDA-MB-231 cells for 48 hours (Fig. 3.2B).

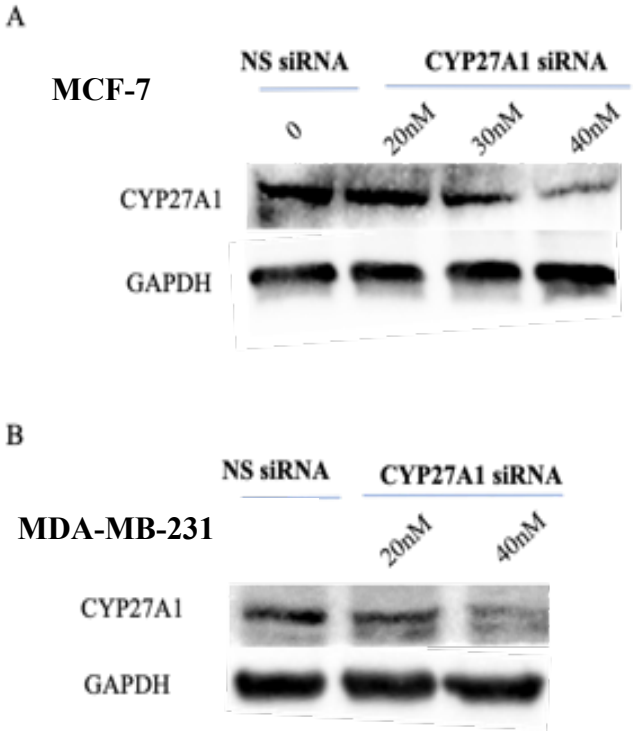


Figure 3. 2: Optimisation of siRNA knockdown of CYP27A1 in MCF-7 and MDA-MB-231 cells

*Cells were treated with siRNA transfection for CYP27A1 knockdown with different concentration of siRNA (20,30, and 40nM) in MCF-7, and (20 and 40nM) in MDA-MB-231. Western blotting analysis confirmed CYP27A1 knockdown in **A)** MCF-7 and **B**) MDA-MB-231. GAPDH were used as loading control. Data representative of one experiment.*

3.4.6 Trans-well migration assay by crystal violet staining

48 h post-treatment with or/without LDL and CYP27A1 siRNA, cells were seeded into transwell inserts to assess migration. The migrated cells were fixed and stained with crystal violet after 24 h for MCF-7 or 6 h for MDA-MB-231 cells. Further protocol details are described in section (2.9).

3.4.7 Statistical analysis

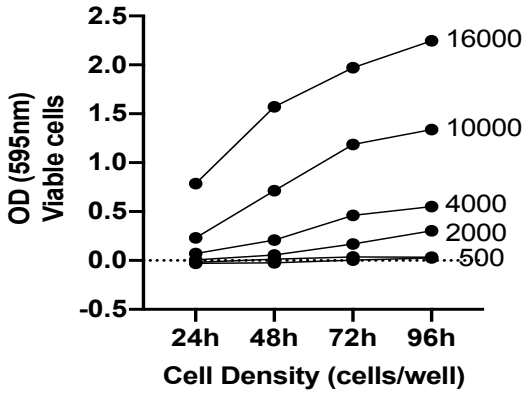
The whole experiments were repeated in triplicate, and also each experiment was repeated three times. Using GraphPad Prism 8.0.1 software for windows (La Jolla, CA, USA), to analyse the data, one-way ANOVA following the least significant difference (LSD) post-hoc test.

3.5 Results

3.5.1 Optimizing a crystal violet (CV) proliferation assay

Figure 3.3 A and B show that at the 2000 to 16000 cell density, the proliferation rate increased in a time-dependent manner up to 96 hours. However, the cell growth rose most sharply at 2000-16000 cells between the time 48 to 96 hours in both MCF-7 and MDA-MB-231 cells (Fig. 3.3A and B respectively). We chose an optimal plating density of 5000-10,000 cells/well for 48hrs. This number of cells lay in between the lowest and highest densities that allowed the cell growth to be increased or decreased depending upon the treatment (Fig. 3.3).

A



B

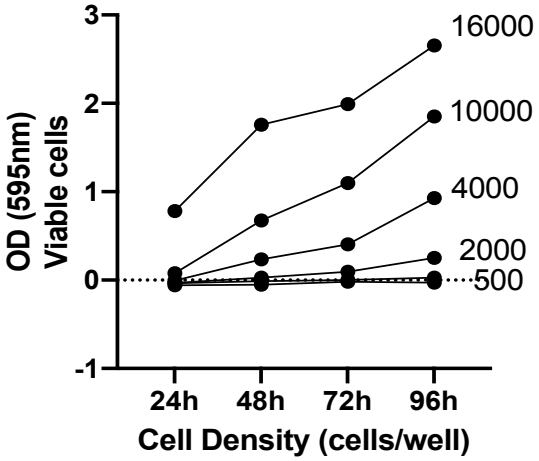


Figure 3. 3: Crystal violet proliferation assay showing the growth rate of MCF-7 and MDA-MB-231 cells

The crystal violet assay was performed to assess the growth rate of A) MCF-7 and B) MDA-MB-231 cells to compare the proliferation rates at different cell densities :500, 2000, 4000, 10000 and 16000cells/well for 24, 48, 72 and 96 hours. Data representative of (n=1).

3.5.2 Effects of LDL on cell proliferation, invasion, and migration

To investigate the appropriate time point for dosing with LDL, we tested different time points: 24 and 48 hours. MCF-7 cells were treated with different concentrations of LDL 0-100µg/ml for 24h and 48h. Figure 3.4 A and B show that the optimum time point for dosing

with LDL was 48 hours, as the growth was greatest at 48h in comparison with the 24h time period (Fig 4.3B).

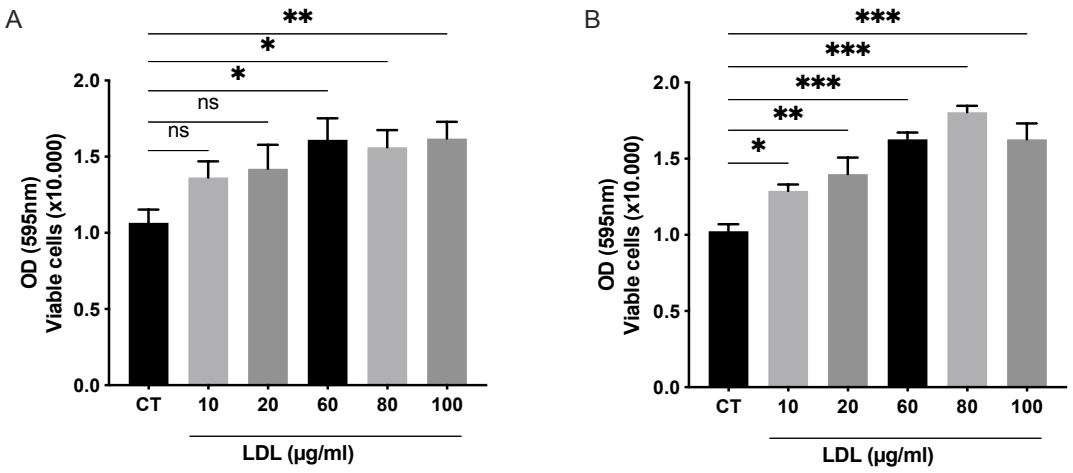


Figure 3. 4: Optimization of cell proliferation at different time points, 24h and 48h in response to LDL treatment, using a CV proliferation assay:

The CV assay was performed to assess the proliferation and viability of MCF-7 cells for A) 24h and B)48h with seeding density at 10,000 cells/well, after being dosed with increasing concentrations of LDL (0-100µg/ml). Absorbance values reflect the number of viable cells that have absorbed the crystal violet dye. Data representative of (n=3).

Next, we investigated the effects of LDL on cell proliferation in a different ERα-positive cell line, T47Ds and two different ERα-negative breast cancer cell lines with different concentrations of LDL 0-100µg/ml. At 48hrs, the proliferation of ERα-positive MCF-7 cells was greatest at 80µg/ml *($p < 0.001$)* LDL in comparison with the control (Fig. 3.4B), whereas with T47D cells, a gradual increase in growth was found with increasing concentrations of LDL from 0-100µg/ml, with a significant increase at 80 and 100µg/ml *($p < 0.001$ for both)* in comparison with the control (Fig. 3.5).

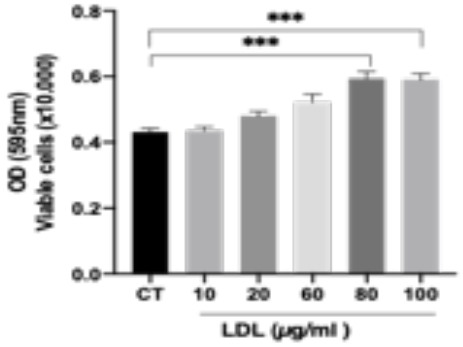


Figure 3. 5: Assessment of proliferation in T47D cells using a crystal violet assay (CV) following LDL treatment

*The CV assay was performed to assess the proliferation and viability of T47D cells with seeding density at 10,000 cells/well after being dosed with increasing concentrations of LDL (0-100µg/ml) for up to 48 hours. Absorbance values reflect the number of viable cells that have absorbed the crystal violet dye. Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

While with the ERα-negative cell lines, figures 3.6 A and B respectively, show a significant change in cell proliferation in response to LDL in MDA-MB-231(Fig. 3.6A) and Hs578T (Fig. 3.6B) cells. There was a dose-dependent increase in growth rate of MDA-MB-231 cells in response to LDL in comparison with the control: a 22.2, 28.5, 31.8, 39.7 and 37.9% increase respectively in growth at 10-100µg/ml with p-values of *($p<0.007$, $p<0.001$, $p<0.001$, $p<0.001$ and $p<0.001$* respectively). A similar pattern of growth was observed in Hs578T with proliferation increasing from 60-100µg/ml LDL; a 16.7% *($p<0.001$),* 28.6% *($p<0.001$)* and 50.7% *($p<0.001$)* increase respectively in comparison to control.

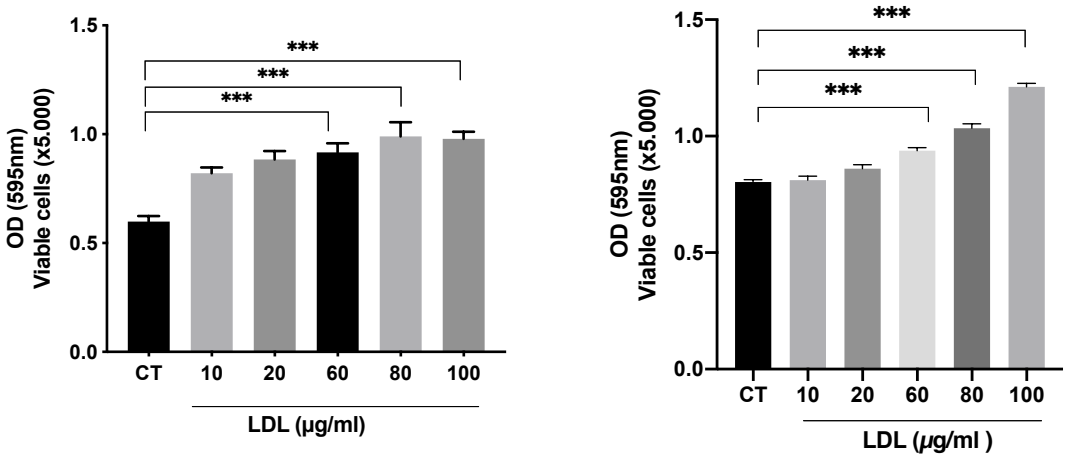


Figure 3. 6: Assessment of proliferation in ERα-negative, A) MDA-MB-231and B) Hs578T cells using a crystal violet assay (CV) following LDL treatment

*The CV assay was performed to assess the proliferation and viability of A) MDA-MB-231 with seeding density at 5,000 cells/well, and B) Hs578T with seeding density at 5000 cells/well after being dosed with increasing concentration of LDL (0-100µg/ml) for 48 hours. Absorbance values reflect the number of viable cells that have absorbed the crystal violet dye. Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

Migration and invasion were significantly increased by LDL treatment (80µg/ml) in both the ERα-positive, MCF-7 cells, with p-value of (*p< 0.001 for migration and p < 0.01 for invasion*) (Fig. 3.7A and B respectively), and ERα-negative, MDA-MB-231 cells, in comparison to control with a p-value of (*p < 0.01 for both)* (Fig. 3.7C and D respectively).

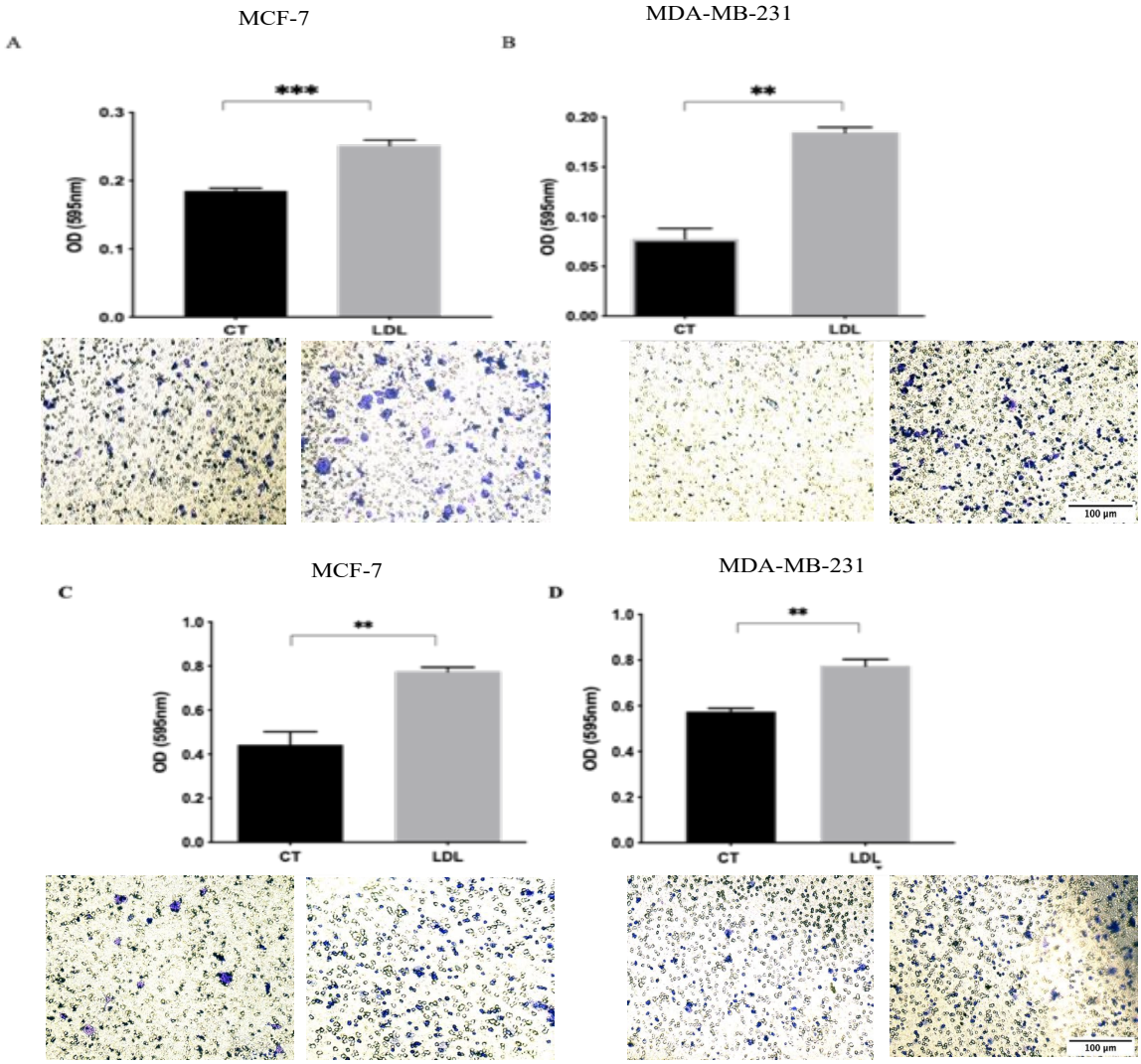


Figure 3. 7: Assessment of migration and invasion in A& B) MCF-7 and C&D) MDA-MB-231 cells using a trans-well migration/invasion assay following treatment with LDL

*Cells were treated with LDL (80µg/ml) for 48 hours. A trans-well assay was used to detect cell migration and invasion. The migrated cells were stained with crystal violet and images were taken (×20 magnification). Quantification of cell migration for A) MCF-7 after 24hours and B) MDA-MB-231 after 6 hours, and cell invasion for C) MCF-7 after 24hours and D) MDA-MB-231 after 6 hours. Quantitative analysis of stained migrated/invaded cells using a microplate reading at an OD at 595 nm. Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test plus least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001), Scale bar represents 100µm.*

3.5.3 Are the effects of cholesterol mediated via 27OHC in breast cancer cells?

As LDL induced proliferation, migration, and invasion in breast cancer cells, we next examined if the effect of LDL actions were through CYP27A1 (is the rate-limiting step in 27-hydroxycholesterol synthesis). MCF-7 and MDA-MB-231 cells were dosed with LDL in the presence or absence of CYP27A1, by using CYP27A1 siRNA.

With MCF-7 cells, LDL increased proliferation and migration ($p < 0.001$ respectively), but with CYP27A1 silenced, these effects were blocked (Fig. 3.8A &B). The western blot shows effective silencing of CYP27A1 in MCF7 cells (Fig. 3.8b-E). Similarly, with MDA-MB-231 cells, LDL promoted cell growth and migration ($p < 0.001$ respectively) and these effects were blocked with CYP27A1 silenced (Fig 3.8a-C&D). The western blot shows effective silencing of CYP27A1 in MDA-MB231(Fig. 3.8b-F).

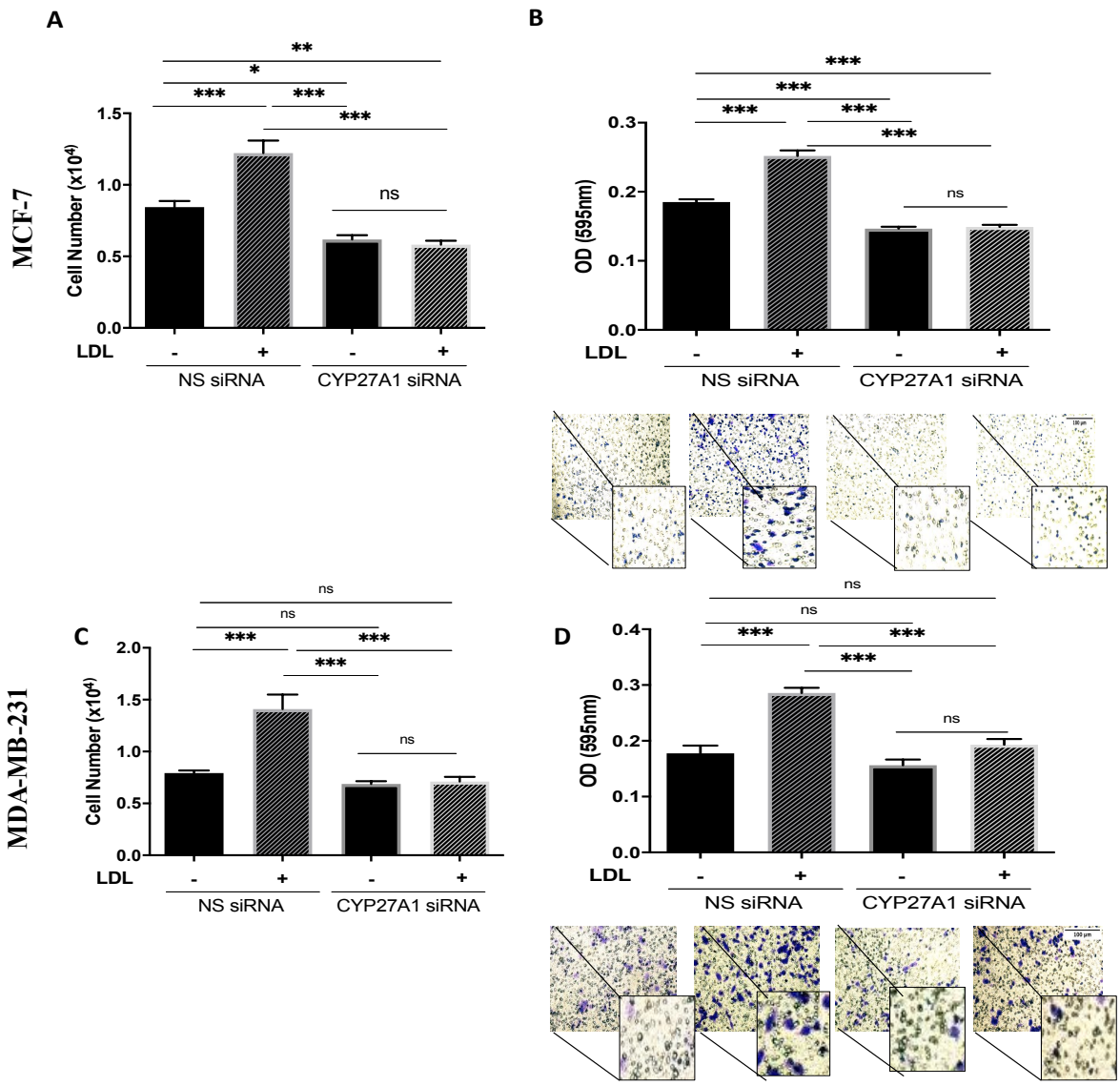


Figure 3. 8a: LDL increases proliferation and migration through 27OHC production in breast cancer cells:

*Using a Muse cell analyser, to assess the cell number for A) MCF-7 and C) MDA-MB-231 after being transfected with CYP27A1 siRNA and non-silencing RNA and dosed with LDL (80µg/ml) for 48 hours. A trans-well assay was used to detect cell migration and invasion after being transfected with CYP27A1 siRNA and dosed with (LDL 80µg/ml). The migrated cells were stained with crystal violet and images were taken (×20 magnification). Quantification of migrated cells were assessed for B) MCF-7 after 24 hours and D) MDA-MB-231 after 6 hours incubation time. Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001), Scale bar represents 100µm.*

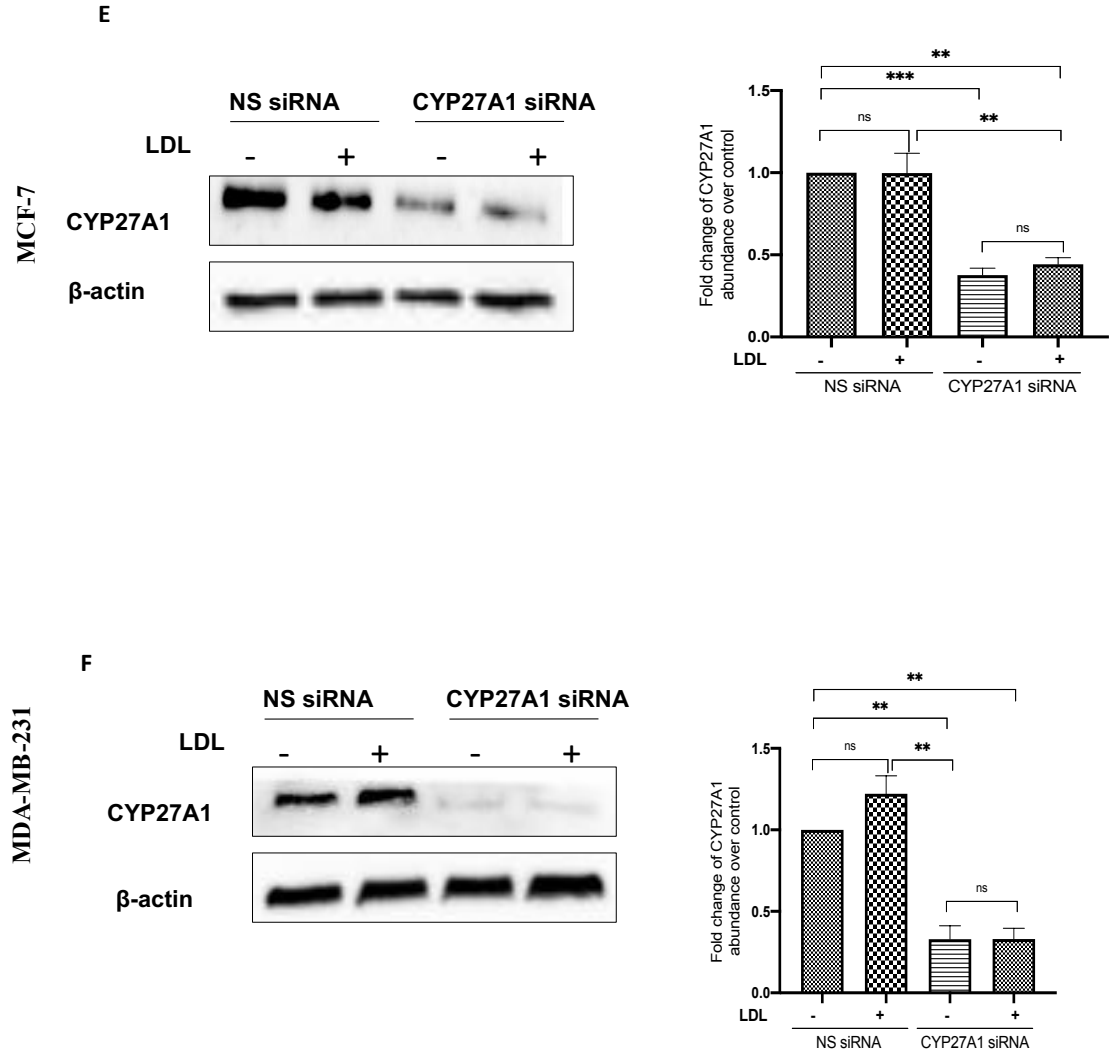


Figure 3. 9b: LDL increases proliferation and migration through 27OHC production in breast cancer cells:

*Western blotting was conducted to show protein abundance of CYP27A1 for E) MCF-7 and F) MDA-MB-231 with or without LDL and CYP27A1 silencing, and relative fold changes of CYP27A1 against loading control β-actin were measured. Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

3.5.4 Cholesterol and its metabolite increased survival in breast cancer cell lines

To investigate the survival effect of LDL and 27OHC breast cancer cells were dosed with a chemotherapy drug, doxorubicin (DOX) to induce cell death. Cells were exposed to DOX treatment (0-20μM) in MDA-MB-231 cells to choose an appropriate concentration, which in this case killed approximately 46.1% of the MDA-MB-231 cells. The optimum dose was 0.5uM for MDA-MB-231 (fig. 3.9B).

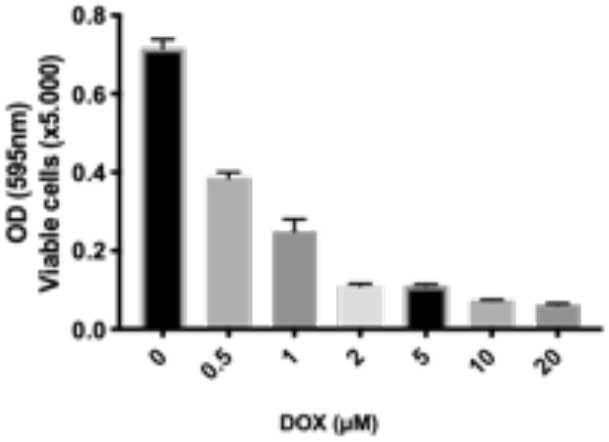


Figure 3. 10: Dose response to doxorubicin for 24hours in ERα -negative cells
*The CV assay was performed to assess the viability of MDA-MB-231 after being dosed with increasing concentration of doxorubicin (0-20μM) for 24 hours. Absorbance values reflect the number of viable cells that have absorbed the crystal violet dye. Data are representative of the mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

Cells were either pre-exposed with 27OHC or LDL for 24 hours, followed by co-exposure with DOX for a further 24hrs. Pre-exposure to 27OHC and LDL significantly reduced doxorubicin-induced cell death. There was a significant increase cell viability and percentage of cell viability respectively with LDL *($p < 0.001$* for both) and 27OHC treatment *($p < 0.001$* for both) (Fig.3.10A &B respectively), and a significant decrease in

the percentage of cell death by 35.91% ($p < 0.001$) with LDL and 30.1% ($p < 0.001$) with 27OHC treatment (Fig.3.10C), in compared to DOX. Doxorubicin is less effective in inducing cell death in the presence of LDL and 27OHC, therefore LDL and 27HC appear to act as survival factors.

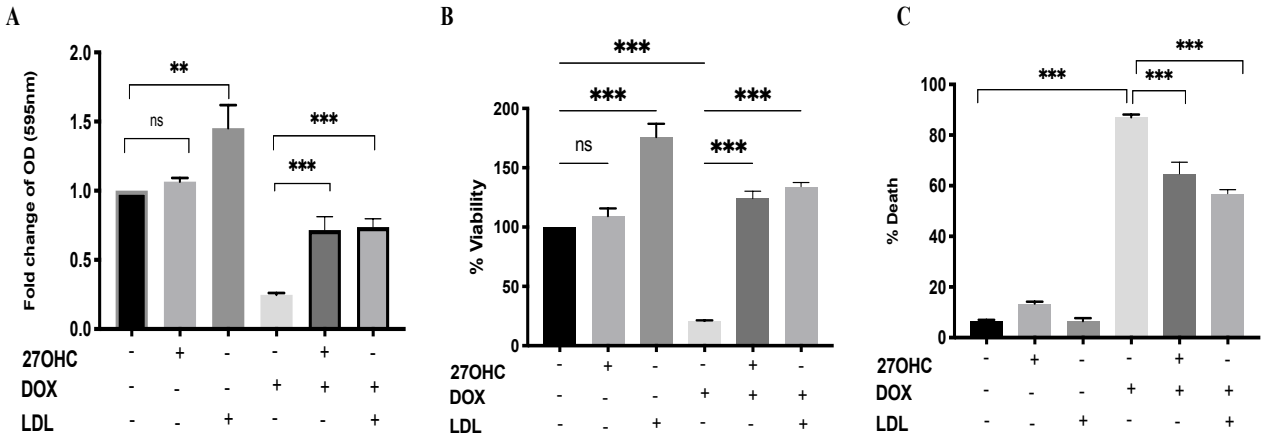


Figure 3. 11: Effects of LDL and 27OHC on the effect of doxorubicin on cell death in the ERα-negative cells:
*Cells were pre-incubated at least 24hrs with 27OHC(0.1μM) and LDL (80μg/ml) before adding doxorubicin (1μM) for 24hrs with/without 27OHC and LDL: A) cell viability and proliferation assay (crystal violet staining assay) detects the cell viability of MDA-MB-231 after 48hrs. Absorbance values reflect the number of viable cells that have absorbed the crystal violet dye. Muse cell counting was employed to detect B) the percentage of cell viability and C) the percentage of cell death in MDA-MB-231 cells. Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

3.5.5 The Cancer Genome Atlas (TCGA) data analysis to assess the expression of CYP27A1 in different types of breast cancer

The Cancer Genome Atlas (TCGA) data analysis was used to assess the expression level of CYP27A1 in different types of breast cancer. The expression level of CYP27A1 mRNA is significantly increased in triple negative breast cancers (TNBC) compared with normal breast tissue (p value $<10^{-6}$), whereas there is no difference found between the HER2-positive group and luminal group compared with normal breast tissue (Fig. 3.11A). The protein expression of CYP27A1 is significantly increased in stage 2 and stage 3 breast cancer groups compared to normal breast tissue, while there is no differences found between stage 1 compared with normal breast tissue (Fig. 3.11B)

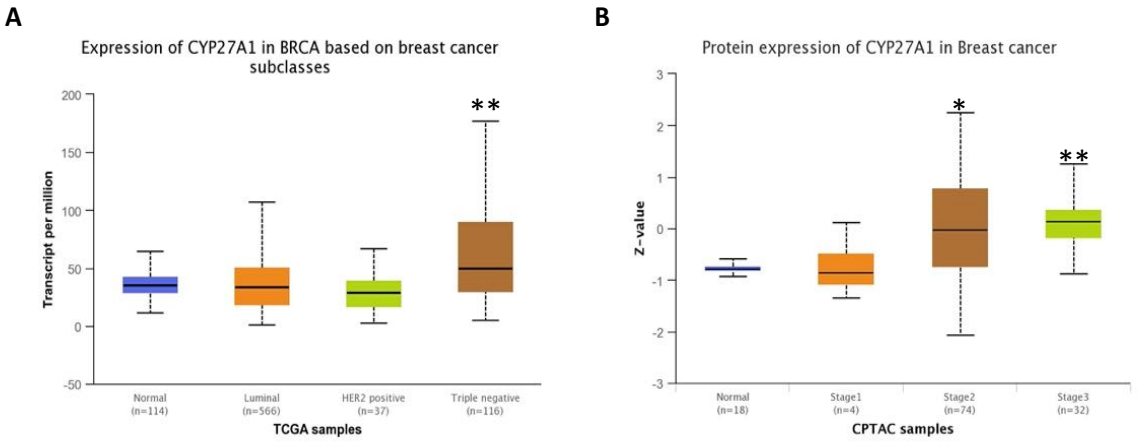


Figure 3. 12: mRNA and protein expression of CYP27A1 in different subtypes of breast invasive carcinoma (BRCA) using the TCGA data:
A) the expression of CYP27A1 in luminal, HER2-positive and TNBC compared to normal breast tissue. **B)** Protein expression analysis using data form Clinical Proteomic Tumor Analysis Consortium (CPTAC) the protein expression of CYP27A1 in different breast cancer stages, stage 1, stage 2 and stage 4, compared to normal. Unpaired two-tailed student's t-test.

3.6 Discussion

Obesity is correlated with an increase in breast cancer-specific mortality in women (Niu *et al.*, 2013). The levels of cholesterol, specifically low-density lipoprotein cholesterol (LDL-C) are frequently up-regulated in women with obesity (Gallagher *et al.*, 2017a). Additionally, it was reported that a high fat diet and raised cholesterol decreased latency and promoted tumour growth and metastasis of breast cancers in mice (Llaverias *et al.*, 2011).

Statins are 3-hydroxy-3-methylglutharyl-coenzyme-A reductase (HMGCR) inhibitors that block the rate limiting step in the production of cholesterol. Recent meta-analyses reported that the use of statins, specifically lipophilic statins in patients with breast cancer show improved overall recurrent-free and cancer- specific survival (Manthravadi, Shrestha and Madhusudhana, 2016; B. Liu *et al.*, 2017). Additionally, similar results with *in vitro* studies found that fluvastatin reduced caveolin-1 (CAV-1) expression, and cerivastatin down-regulated cyclin D1 in breast cancer cells (Denoyelle *et al.*, 2001; Salis *et al.*, 2014)

We found that LDL drives the proliferation of ERα-positive and ERα-negative cells, and this is consistent with a previous study examining the importance of the LDL-R in the growth of TNBCs (Gallagher *et al.*, 2017a). Although the differences in the optical density (OD) readings are small, the fold change is comparable to that seen with cell counting.

A correlation between the circulating levels of 27OHC and hypercholesterolemia has been reported (Brown and Jessup, 1999; Burkard *et al.*, 2007; Wu *et al.*, 2016a). In our laboratory, we found that LDL increased the proliferation of ERα-positive and ERα-negative cells and this was inhibited when CYP27A1 was silenced using siRNA, suggesting that cholesterol was metabolised to 27OHC that was responsible for increasing

the growth of the breast cancer cells. The phenotype that we observed has been confirmed by others (Nelson *et al.*, 2013a; Wu *et al.*, 2013). It has been reported that statin treatment significantly decreased serum 27OHC and may deregulate CYP27A1 expression in breast cancer (Kimbung *et al.*, 2017a). According to our analysis of the "The Cancer Genome Atlas (TCGA)" data show that the expression of the enzyme that converts cholesterol into 27OHC, CYP27A1, is the same in ER or HER2 positive breast tumour tissue in comparison with normal breast tissue, but its expression is significantly upregulated in TNBC tumour samples in comparison with normal breast tissue. Moreover, the protein expression of CYP27A1, is significantly increased in stage 2 and stage 3 breast cancer group in comparison with normal breast tissue, suggesting a potential role of disrupted 27OHC homeostasis in breast cancer development. This is consistent with previous work showing increased expression of CYP27A1 enzyme in higher grade tumours (Kimbung *et al.*, 2017a). Another study reported that a high fat diet significantly increased circulating levels of both total cholesterol, 27OHC and promoted tumour growth and this effect was decreased by treatment with a CYP27A1 inhibitor in mice with ER-positive breast cancer compared to mice on a control diet, and is thought to be mediated through the estrogen receptor (Nelson *et al.*, 2013a). Exogenous 27OHC supplementation increased lung cancer cell proliferation (Hiramitsu *et al*., 2018), suggesting 27OHC signalling can mediate the effects of cholesterol.

We have shown that the effects of cholesterol on the proliferation of ER-positive and ER-negative breast cancer cells involves its conversion to 27OHC. It is important to mention that other metabolites of cholesterol, such as 25-hydroxycholesterol (25OHC), may also be involved and we have not yet explored how cholesterol treatment may alter other such

metabolites. 25OHC is generated from cholesterol via CH25H enzyme, CYP27A1 and cytochrome P450 3A4 (CYP3A4) and can be created by free radical oxidation (Yamauchi and Rogers, 2018).

Interestingly, 27OHC promoted breast cancer metastasis and this effect was decreased with a CYP27A1 inhibitor in mice with ER-positive breast cancer compared to mice on a control diet (Nelson *et al.*, 2013b). In our laboratory, we found that LDL promoted the migration and invasion of ERα-positive and ERα-negative cells and this was inhibited when CYP27A1 was silenced using siRNA, suggesting that the role of dietary cholesterol on migration and invasion requires the conversion of cholesterol to 27OHC via CYP27A1. In support of the mechanism that the 27OHC signalling pathway mediates the role of cholesterol, it was found that exogenous treatment with 27OHC induced lung cancer cell metastasis (Hiramitsu *et al.*, 2018). In the next chapter we examine the effect of exogenous 27OHC on proliferation, migration, and invasion to examine the role of endogenously produced 27OHC. 27OHC is an important biochemical mediator of the metastatic effects of high level of cholesterol through its interaction with immune cells γδ-T cells and polymorphonuclear-neutrophils (PMNs) and decreasing CD8+T cells (Baek *et al.*, 2017). Although the exact signalling pathways involved are not yet known, 27OHC has been found to interact with nuclear receptors as the ERs and LXRs. Additionally, cholesterol was found as a ligand for the estrogen-related receptor alpha (ERRα), and it has been suggested that 27OHC might also interact with this receptor(Wei *et al.*, 2016). In next chapter, we investigate the action of exogenous 27OHC on migration and invasion and through which receptors these actions might be mediated.

Anti-cancer, DNA-damaging agents, including doxorubicin have been widely used to treat different types of cancer. Recently, it was found that doxorubicin significantly downregulates HMG-CR and this leads to a reduce in the level of the EGFR, and thus enhances doxorubicin-induced cell death. In addition, knockdown of HMG-CR in the presence of DOX promoted PARP cleavage, which is an indicator of consequent apoptosis and caspase-3 activation (Yun *et al*., 2019). Consistent with these observations, we found that doxorubicin was less effective in inducing cell death in the presence of 27OHC or LDL, suggesting they can act as survival factors in MDA-MB-231, ERα-negative breast cancer cell lines.

These results are consistent with an animal model treated with doxorubicin, which demonstrated that the tumour growth was faster in mice fed with a high-cholesterol diet than on a normal diet and HMG-CR expression likely to be inversely associated with decreased overall survival and is increased in tumour than in normal tissues (Yun *et al*., 2019). In *vitro,* 27OHC and 7-ketocholesterol increased doxorubicin resistance in MCF-7 breast cancer cells and this might be mediated through the upregulation of P-glycoprotein in an ERα- and mTOR-dependent pathway (Wang *et al.*, 2017). Our data suggest that cholesterol levels may influence doxorubicin sensitivity.

3.7 Conclusion

In summary, our data support the clinical studies suggesting a link between obesity and high cholesterol with an increased risk of breast cancer progression: we found that LDL promotes cell proliferation, migration and invasion and chemo-resistance and that this may involve the oxysterol metabolite, 27OHC.

Chapter 4.

The effect of 27-Hydroxycholesterol on breast cancer cell proliferation, migration, and invasion and on levels of EMT markers

4.1 Introduction

Cholesterol molecules generate oxidized metabolites, defined cholesterol oxidation products (COP) or oxysterols, that mediate different signal transduction cascades. At very low concentrations, oxysterols play a vital role in the human body, mediating many physiological functions; they regulate cholesterol metabolism, influence signalling molecules such as MAPK (Caihua Wang *et al.*, 2017), INSIG SREBP (LP *et al.*, 2005b), SCAP and the activity of some membrane proteins such as ATP-binding cassette transporters, ABCA1, ABCB1, Na+/ K+ ATPase to affect membrane fluidity (Ouvrier *et al.*, 2009). Recent evidence shows that oxysterols influence cancer progression and carcinogenesis (Kloudova, Guengerich and Soucek, 2017). The most abundant oxysterol in plasma is 27OHC that is a SERM (He and Nelson, 2017) and is also a modulator of LXR (Fu *et al.*, 2001). In an acidic pathway through hydroxylation, 27OHC is synthesised from cholesterol by the mitochondrial resident cytochrome P450 enzyme, CYP27A1 (Russell, 2000). 27OHC is a regulator of LXR activity in ERα-positive breast cancer cells, resulting in the arrest of cell cycle progression and a reduction of cell proliferation (Wu *et al.*, 2016a).

The bioavailability of 27OHC at the tumour site can be totally different from its level in the plasma, and this may be more critical in the context of tumour progression. The abundance of 27OHC in ERα-positive breast cancer cells is 2.5- 3-fold higher than in normal breast cells. The concentration of 27OHC in healthy human plasma is 0.22 to 0.60μM and 50–90% of 27OHC is esterified and thus, unesterified plasma levels around 10^{-8}M, and 10^{-8}M was the threshold concentration for activation of breast cancer cell growth (Burkard, Rentsch and Von Eckardstein, 2004; Wu *et al.*, 2013). The relative

contributions of the bioavailability of 27OHC at the tumour site is also regulated by intra-tumoral expression level of the CYP27A1 enzyme that catalyses production of 27OHC from cholesterol (Kimbung *et al.*, 2017a). High expression of CYP27A1 is associated with a higher grade of tumour in breast cancer patients (Kimbung *et al.*, 2017a). Recently, CYP27A1 expression has been identified as an independent prognostic factor for overall and recurrence-free survival in ER-positive breast cancer women > 50 years of age (Kimbung *et al.*, 2017a).

A recent report indicated that 27OHC activated the STAT signalling pathway that plays an important role in cell proliferation and migration (Jiao *et al.*, 2020). Furthermore, oxysterols have been found to induce the activation of the JAK/STAT signalling cascade through LXR-dependent and LXR-independent mechanisms (Nelson *et al.*, 2013b). Several studies have described that activation of the ER is increased by 27OHC in breast cancer cells, which culminates in increased cell proliferation and consequently promoted ERα-positive breast cancer progression and migration (DuSell *et al.*, 2008; Wu *et al.*, 2013; Nguyen *et al.*, 2015; He and Nelson, 2017). Interestingly, 27OHC increased migration and invasion not only in ERα-positive breast cancer cells, but also in ERα-negative cell lines that involved STAT3 activation (Shen *et al.*, 2017).

Currently, the exact mechanism of action of 27OHC in ERα-positive and ERα-negative breast cancer cells and the role of these metabolites in breast cancer cell proliferation, migration and invasion remains to be elucidated.

4.2 Aims

In this chapter, we aimed to assess the effect of 27OHC on cell proliferation, migration, and invasion in breast cancer epithelial cell lines.

4.3 Specific aims

Aim 1: To examine the effect of 27OHC on cell proliferation, migration, and invasion in both ERα-positive and ERα-negative breast cancer epithelial cell lines.

Aim 2: To determine the roles of ERα in the effects of 27OHC on cell migration proliferation and invasion in MCF-7 cells.

4.4 Methods

4.4.1 Cell culture:

The human breast cancer cell lines ERα-positive; MCF7 and T47D and ERα-negative; MDA-MB-231 and Hs578T were obtained from ATCC (Teddington, Middlesex, UK). All cell lines were cultured as described previously in section 2.1.1.

4.4.2 Cell counting:

For cell proliferation assays, cells were incubated in growth media DMEM for 24 hours, before being changed to serum-free media for a further 24 hours. The cells were then treated with different concentrations of 27OHC 0-0.1μM. Crystal violet proliferation assay was used to determine the viability and proliferation of MCF-7 and MDA-MB-231, as outlined in section (2.6).

4.4.3 Western blot

Western blot analysis was observed as described previously (2.10). In brief, protein cell lysates (30 μg), were allocated in SDS-PAGE gel, transferred to nitrocellulose membrane and immunoblotted with the targeted antibodies: anti-estrogen receptor α (ERα) (1:1000; Santa Cruz Technology), anti-estrogen receptor β (ERβ)(1:1000; Thermo Fisher), fibronectin and vimentin (1;1000; BD Transductio), Low density Lipoprotein-Receptor (LDL-R) (1;1000; Merck Millipore), anti- Liver-X-Receptor (LXR-β) (1;1000;Cell Signalling Technology), GAPDH (1:5,000; Millipore) and β-actin (1:10,000; Sigma-Aldrich). After that the membranes were incubated with particular secondary antibodies (anti Mouse or Rabbit) conjugated to peroxidase, next proteins were detected by Clarity ECL substrate and quantified using Image J software.

4.4.4. Targeted estrogen receptor α (ERα) gene knockdown, using siRNA knockdown

For experiments investigating the potential interactions of 27-OHC with the estrogen receptor, we investigated the effect of 27OHC in the presence or absence of ERα. As previously established in our lab by (Zielinska *et al.*, 2018), the optimum concentration of siRNA for silencing the ERα is 20nM for 48 hours in ERα–positive cells . Transfection with siRNA was performed as described previously in section (2.7).

4.4.5. Trans-well migration assay by crystal violet staining

48 h post-treatment with or/without 27OHC and ERα siRNA, cells were seeded into transwell inserts to assess migration. The migrated cells were fixed and stained with crystal violet after 24 h for MCF-7 or 6 h for MDA-MB-23. Further protocol details mentioned in section (2.9).

4.4.6. Statistical analysis

The whole experiments were repeated in triplicate, and also each experiment was repeated three times. Using GraphPad Prism 8.0.1 software for windows (La Jolla, CA, USA), to analyse the data, one-way ANOVA following the least significant difference (LSD) post-hoc test.

4.5 Results

4.5.1 Effects of 27OHC on cell proliferation

In the previous chapter, we found that LDL promoted migration and invasion of breast cancer cells and that this was inhibited when CYP27A1 was silenced, suggesting that dietary cholesterol requires the conversion of cholesterol to 27OHC via CYP27A1 to exert its effects. To investigate this further, we examined the effect of exogenous 27OHC on proliferation, migration, and invasion. We found that at 24hrs, the proliferation of ERα-positive MCF-7 cells was greatest at 1μM 27OHC in comparison to control (Fig. 4.1A), whereas at 48hrs, peak growth in response to 27OHC occurred at 0.1μM *($p < 0.001$)* in comparison to the control (Fig. 4.1B).

Results from the MCF-7 cells indicated that the ideal incubation time for dosing with 27OHC in ERα-positive cell lines was 48 hours. To confirm the results obtained using the CV assay, the effects of 27OHC on MCF-7 cell proliferation was also performed by MUSE counting assay at the same doses over 48hrs. A similar pattern of growth was observed with a peak in cell proliferation found at 0.1μM *($p < 0.001$)* in comparison to control (Fig. 4.1C). To confirm the results, we used another ERα-positive cell lines T47D cell and the same pattern was observed with a peak cell growth in response to 27OHC at 0.1μM *($p < 0.001$)* in comparison to control, at 48hrs (Fig. 4.2).

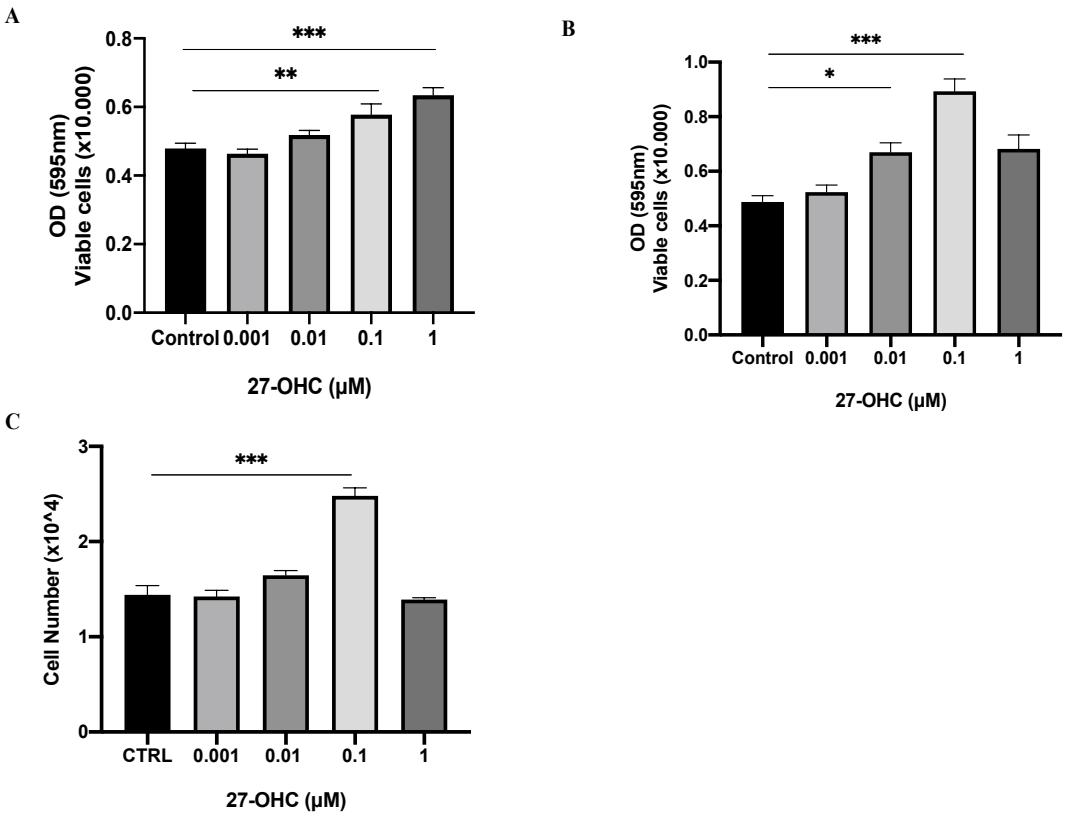


Figure 4. 1: Assessment of proliferation in MCF-7 cells using a crystal violet assay (CV) and MUSE cell counting following 27OHC treatment:

*The CV assay was performed to assess the proliferation of A) MCF-7 treated with increasing concentration of 27OHC (0- 1µM) for up to A) 24 and B) 48 hours. Absorbance values reflect the number of viable cells that have absorbed the CV dye. Muse cell counting was employed to confirm the CV assay results shown in C. C) Number of MCF-7 cells after being dosed with increasing concentrations of 27OHC (0- 1µM) for up to 48 hours. Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

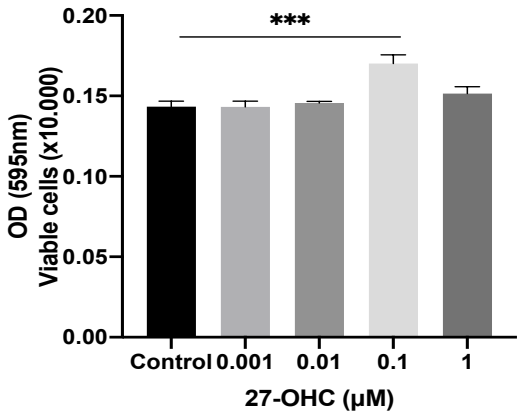


Figure 4. 2: Assessment of proliferation in T47D cells using a crystal violet assay (CV) following 27OHC treatment

*The CV assay was performed to assess the proliferation of T47D treated with different concentration of 27OHC (0-1µM for 48 hours, was assessed using a CV assay. Absorbance values reflect the number of viable cells that have absorbed the CV dye. Data representative of mean ±SEM (n=3). P-value were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

With the ERα-negative cell lines, figures 4.3A and B respectively, no significant changes in cell proliferation in response to 27OHC in either MDA-MB-231(Fig. 4.3A) or Hs578T (Fig. 4.3B) cells, were observed in comparison to control.

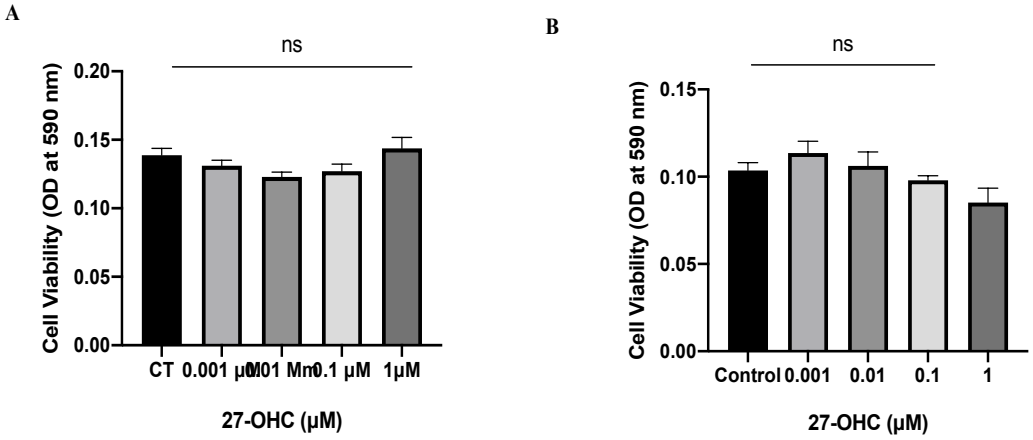


Figure 4. 3: Assessment of proliferation in MDA-MB-231 cells using a crystal violet assay (CV) following treatment with 27OHC

*A) Cell proliferation of MDA-MB-231 and B) Hs578T treated with different concentration of 27OHC (0-1µM for 48 hours and assessed using a CV assay. Absorbance values reflect the number of viable cells that have absorbed the CV dye. Muse cell counting was employed to confirm the CV assay results. Data representative of mean ±SEM (n=3). P-value were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

4.5.2 Effects of 27-hydroxycholesterol on cell migration, invasion, and the abundance of EMT markers

Migration and invasion were significantly increased by 27OHC (0.1µM) in both the ERα-positive, MCF-7, ($p < 0.001$ for both) (Fig. 4.4 A+C) and ERα-negative, MDA-MB-231 cells (Fig. 4.4B and D) ($p < 0.001$for both).

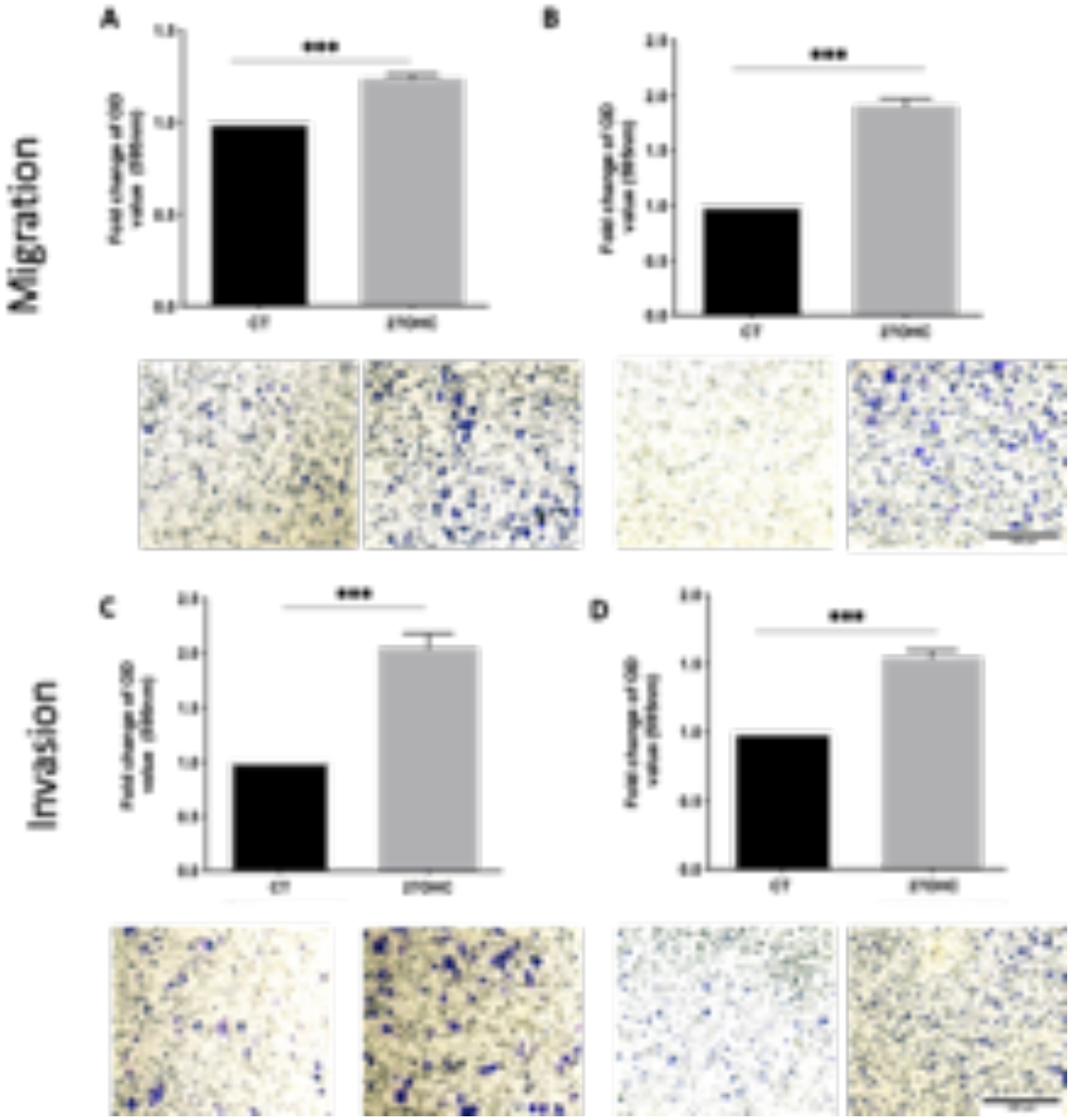


Figure 4. 4: Assessment of migration and invasion in MCF-7 and MDA-MB-231 cells using a trans-well migration/invasion assay following treatment with 27OHC

*Cells were dosed with different concentration of 27OHC (0.1μM) for 48 hours, a trans-well assay was used to detect cell migration and invasion. The migrated/ invaded cells were stained with CV for A) MCF-7 after 24hours and B) MDA-MB-231 after 6 hours, and invaded cell for C) MCF-7 and D) MDA-MB-231 and images were taken (×20 magnification). Quantitative analysis of stained migrated/invaded cells were assessed by using a microplate reading at an OD at 595 nm. Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

The effects of LDL, on migration and invasion were assessed in chapter 3 and in this chapter, we examined the effects of 27OHC and LDL on the abundance of EMT markers in both ERα-positive and ERα-negative breast cancer cell lines. After treatment with 27OHC and LDL for 48hrs in MCF-7 cells, we found a significant increase in fibronectin ($p < 0.001$ *for both*) and a decrease in E-cadherin ($p < 0.001$ *for both*) (Fig. 4.5A), while in MDA-MB-231 cells, we found a significant increase in both fibronectin ($p < 0.001$ *for both*) and vimentin ($p < 0.01$ *for 27OHC and* $p < 0.05$ *for LDL*) (Fig. 4.5B).

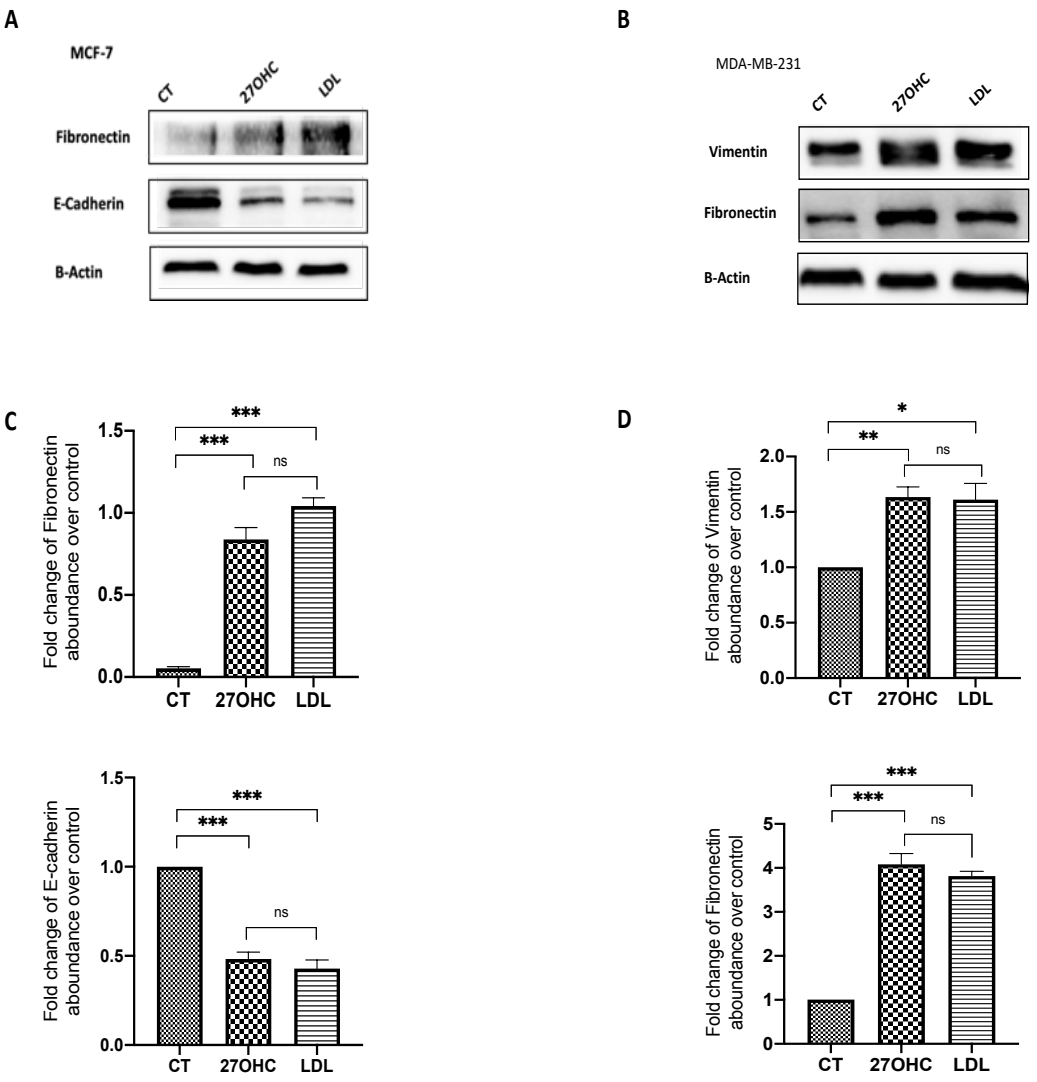


Figure 4. 5: Abundance of EMT markers in MCF-7 and MDA-MB-231 cells using western blotting following 27OHC and LDL treatment.

*Western blotting was conducted to show protein abundance of EMT markers fibronectin and E-cadherin in **A)** MCF-7 and fibronectin and vimentin in **B)** MDA-MB-231 with or without 27OHC (0.1μM) and LDL (80μg/ml) treatment for 48 hours. **C&D)** Densitometry analysis of fold changes of fibronectin, E-cadherin, and vimentin expression relative to B-actin normalized to control. Data representative of mean ±SEM (n=3). P-value were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

4.5.3. Effects of 27-hydroxycholesterol on cell proliferation in the presence or absence of the ERα

To address the potential interactions of 27OHC with the estrogen receptor, we investigated the effect of 27OHC in the presence or absence of the ER. As previously established in our laboratory, the optimum concentration of siRNA for silencing the ERα is 20nM for 48 hours in ERα-positive cells (Zielinska *et al.*, 2018). Figure 4.6A shows that 27OHC treatment increased MCF-7 proliferation *($p < 0.001$)* in comparison to control, whereas with knockdown of ERα, 27OHC was unable to increase cell growth in comparison to control. Effective silencing of the ERα was shown by Western blotting (Fig. 4.6B & C). 27OHC treatment increased the abundance of ERα significantly as shown in figure 4.6B and C. These data suggest that 27OHC interacts with the ERα to promote breast cancer cell proliferation.

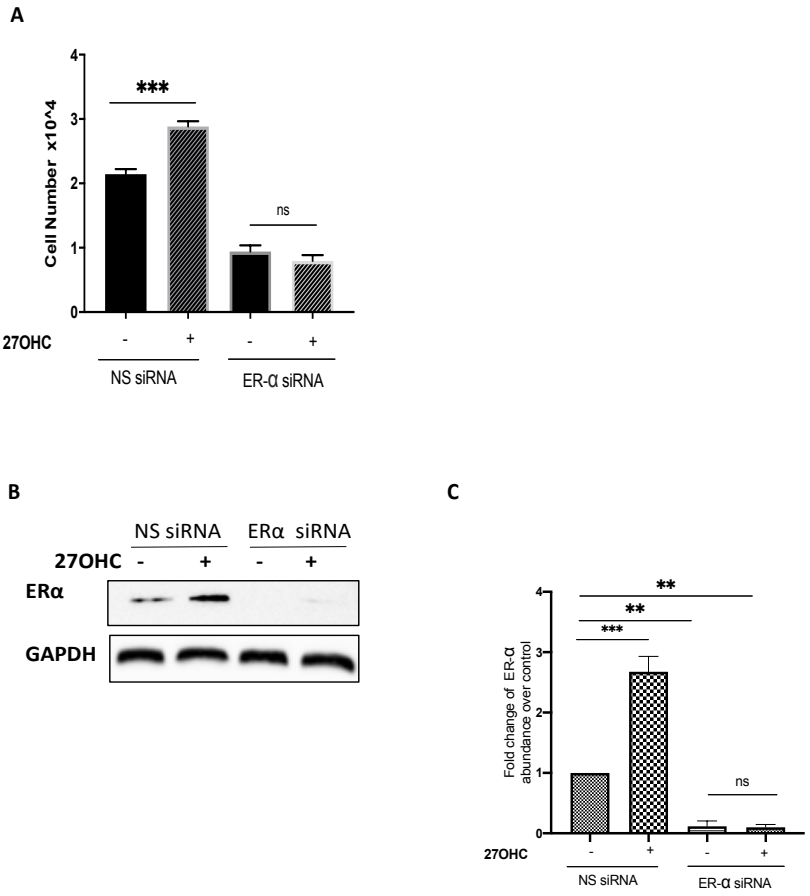


Figure 4. 6: Effects of 27OHC on proliferation of MCF-7 cells in the presence or absence of the ERα.

*Muse cell counting was employed to assess the cell number of A) MCF-7 after being transfected with ERα siRNA (20nM) and non-silencing RNA (20nM) and dosed with 27OHC (0.1μM) for 48 hours. B) Western blot analysis of the ERα. C) Densitometry analysis of fold changes of ERα levels relative to B-Actin normalized to control. Data representative of mean ±SEM (n=3). P-values were determined using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

4.5.4. Effects of 27-hydroxycholesterol on cell migration in the presence or absence of the ERα

We silenced the ERα using siRNA in MCF-7 cells treated with 27OHC and then assessed changes in migration and invasion. The ability of 27OHC to increase cell migration and invasion was unaffected with the ERα silenced (Fig. 4.7A and B), suggesting that 27OHC-induced migration and invasion of MCF-7 cells was not mediated via the ERα. Effective silencing of the ERα was shown by Western blotting (Fig. 4.7C-D).

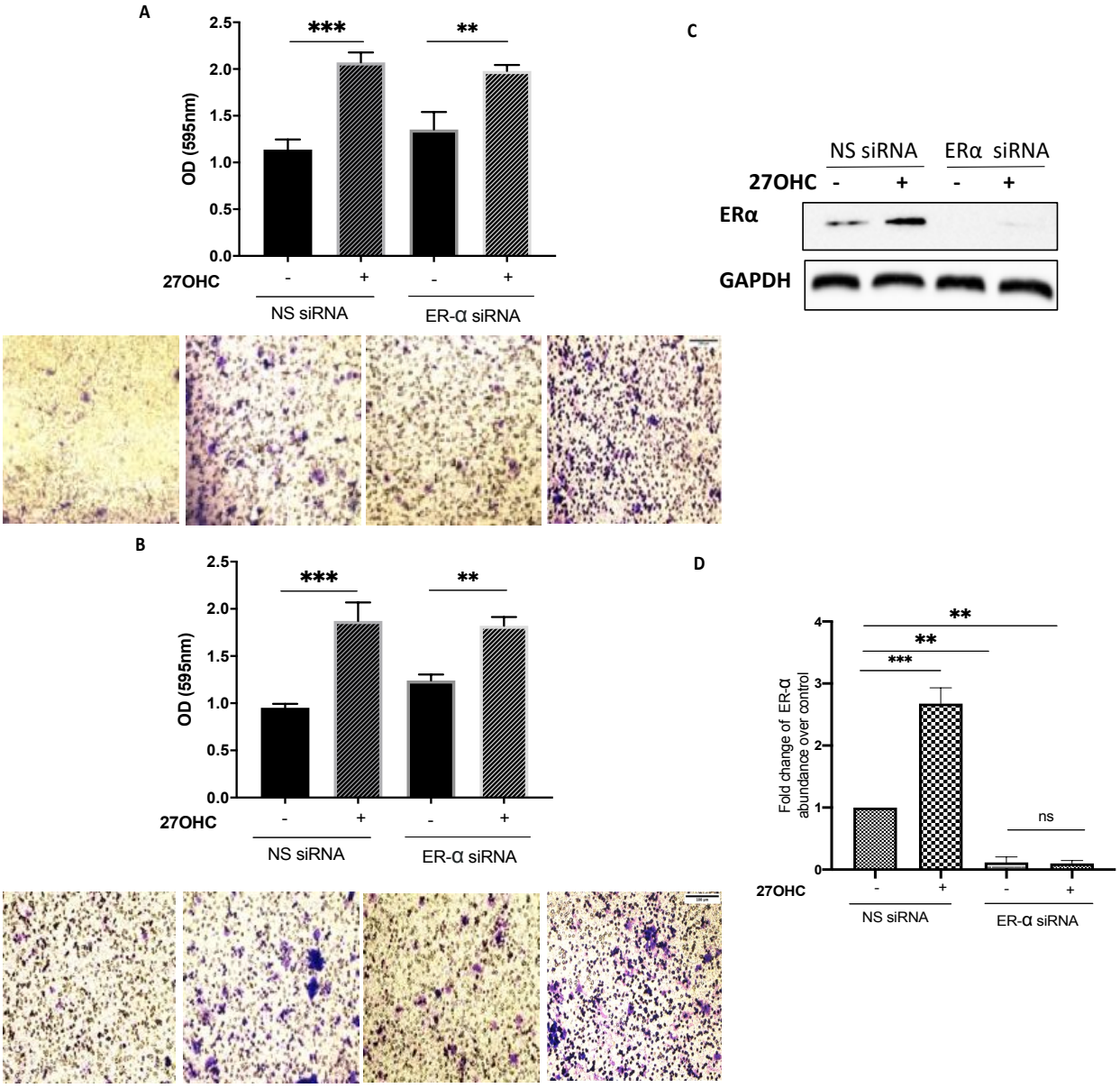


Figure 4. 7: Effects of 27OHC on migration and invasion of MCF-7 cells in the presence or absence of the ERα.

*Trans-well assay was used to detect cell migration for A) MCF-7 after 24 hours and B) invasion after24 hours after being transfected with ERα siRNA and dosed with 27OHC 0.1μM. The migrated cell was stained with crystal violet and images were taken (×20 magnification). Quantitative analysis of stained migrated/invaded cells using a microplate reading at an OD at 595 nm. C) Western blotting analysis of ERα knockdown and overexpression. C) Densitometry analysis of fold changes of ERα expression relative to B-actin normalized to control. Data representative of mean ±SEM (n=3). P-value were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001). Scale bar represents 100μm.*

4.5.5. Role of other nuclear receptors in mediating the effects of 27OHC on cell migration and invasion

In MCF-7 cells, we showed that the ERα only mediated the effects of 27OHC on cell proliferation but not its impact on cell migration and invasion. To try to determine which receptor might be involved, we knocked down the ERα using siRNA in ERα-positive cells and assessed the abundance of alternative receptors to which 27OHC can bind: *LXR-β*, ERβ and the LDL-R.

Our preliminary data suggested that silencing ERα only increased the abundance of ERβ in ERα-positive cell lines, in comparison to LDL-R and LXRβ expression (Fig. 4.8 A). This suggested that perhaps the LDL-R and *LXR*- were not involved, but that ERβ may play a role in the effects of 27OHC when the ERα was silenced.

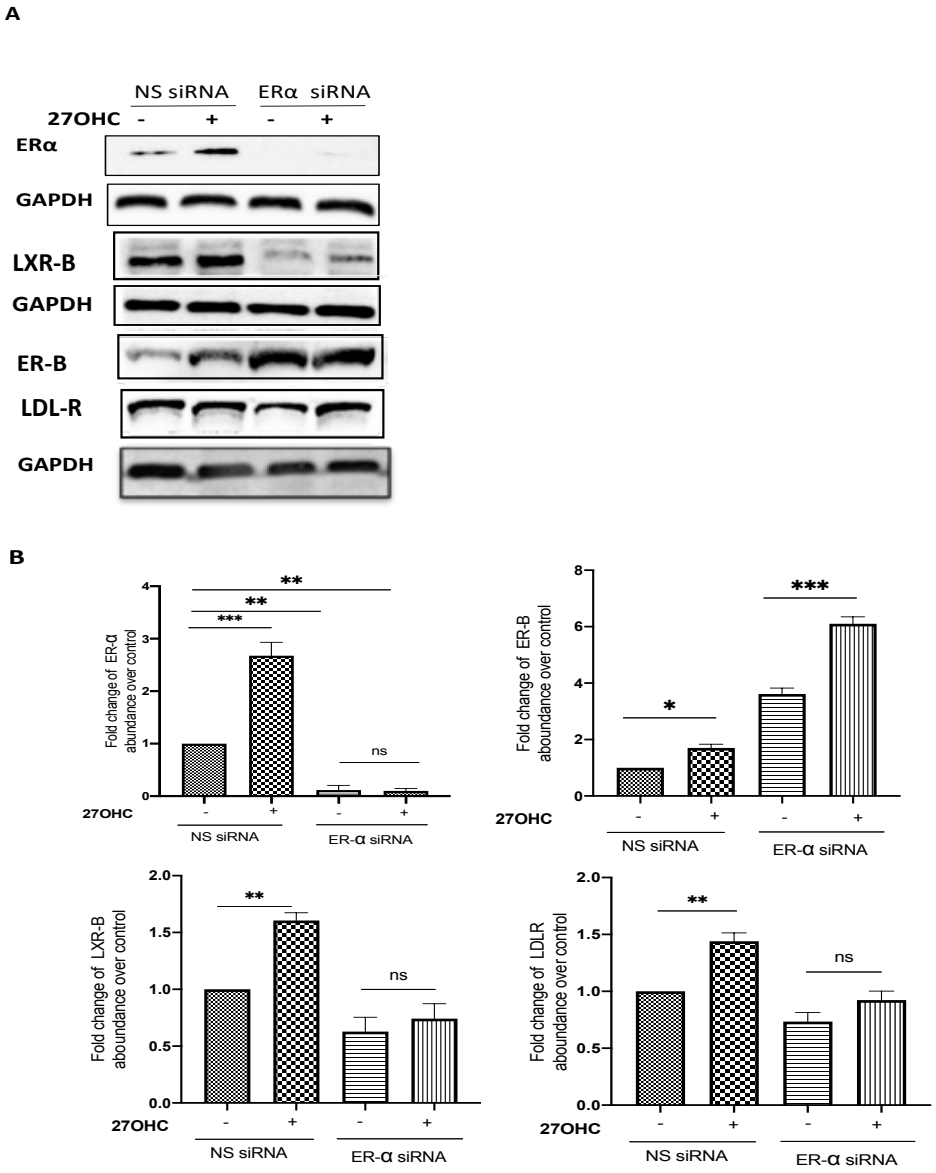


Figure 4. 8: Expression of nuclear receptors in MCF-7 cells in the presence or absence of the ERα using western blotting following 27OHC treatment.

*A) Western blotting analysis was performed to assess changes in the protein levels of ERα, LXR-β, ERβ and LDL-R. ;B) Densitometry analysis of fold changes of ERα, LXR-β, ERβ and LDLR expression relative to β-actin and GAPDH normalized to control. Data representative of mean ±SEM (n=3). P-value were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

4.6 Discussion

Estrogen is recognised as the main ligand of the ER that promotes ERα-positive breast tumour growth (Huang, Warner and Gustafsson, 2015a). Estrogen binds and activates the ER (encoded by ESR1) that then translocate to the nucleus, to regulate the expression of genes that are linked with cell proliferation and survival (Lipovka and Konhilas, 2016).

Cholesterol and its metabolites are known risk factors for breast cancer progression (Mcdonnell *et al.*, 2014) and it has been observed previously that 27OHC behaves as a selective estrogen receptor modulator (He and Nelson, 2017). Several studies indicate the role of cholesterol metabolites in enhancing tumour growth and invasion (DuSell *et al*., 2008; Wu *et al*., 2013; Nguyen *et al*., 2015; He and Nelson, 2017). We show that 27OHC can promote cell growth in ERα -positive cell lines, but not in ERα-negative cell lines. This proliferative effect of 27OHC was mediated via the ERα.

The proliferative effects of 27OHC on breast cancer is well established in the MCF-7 cell line (DuSell *et al.*, 2008), in mice (Nelson *et al.*, 2013a; Baek and Nelson, 2016) and in human breast tumour tissue (Shahoei and Nelson, 2019). Also, a recent report has proposed that 27OHC promotes MDM2-mediated p53 (tumour suppressor protein p53) inactivation through ERs which subsequently results in cell proliferation (Raza *et al.*, 2015). Furthermore, 27OHC was found to have no effect on the proliferation of ER-negative breast cancer cells, SKBR3 and MDA-MB-231 (Cruz *et al.*, 2010). The same observations were found for endometrial cancer cell lines with an effect on the proliferation of RL95 and Ishikawa cells but not on low or poorly ERα -positive cell lines, MFE280 cells, agreeing with stated ER expression in these cell lines (Gibson *et al.*, 2018). This evidence

is consistent with our findings suggesting that 27OHC only exerts a proliferative effect in ERα-positive breast cancer cell lines.

Furthermore, we found that silencing the ERα blocked the proliferative effect of 27OHC, in-line with the evidence suggesting a role for cholesterol metabolites in promoting the growth of ERα-positive breast cancers through acting as endogenous SERMS (DuSell *et al.*, 2008; He and Nelson, 2017; Starkey *et al.*, 2018).

Levels of 27OHC correlate with high grade breast cancers (Nelson *et al.*, 2013b) and have been shown to have metastatic actions (Baek and Nelson, 2016). Here, we show that 27OHC promotes breast cancer cell migration, invasion and modulates EMT markers in both ERα-positive and -negative cell lines. 27OHC had previously been shown to interact with ERs and LXRs to induce migration and invasion of breast cancer cell (Shen *et al.*, 2017). This effect might be due to the metastatic actions of 27OHC reported to be mediated through the activation of STAT-3 by increasing matrix metalloproteinase 9 (MMP9) and generating EMT in ERα-positive (MCF-7 and T47D) and -negative cell lines (MDA-MB-231) (Shen *et al.*, 2017). These studies are consistent with our finding suggesting that 27OHC induces migration/invasion and EMT in both ER-positive and ER-negative breast cancer cell lines.

The phosphorylation of ERα in MCF-7 cells has been suggested to induce cell migration and invasion, suggesting the effect of 27OHC on cell migration and invasion may be mediated through ERα in MCF-7 (Huderson *et al.*, 2012). However, we found that the ERα does not mediate the migration and invasion induced by 27OHC in ERα-positive breast cancer cells. However, there are several other nuclear receptors that may play a crucial role in oxysterol actions, such as, LXRs, ERs and the LDLR. 27OHC acts as a SERM and

oxysterols have been shown to indirectly modulate ERs through interaction with the nuclear receptor *LXR-β* (Ishikawa *et al.*, 2013)(Gallagher *et al.*, 2017a). We elucidated that the proliferative effects of 27OHC involved the ERα.

Caveolae have a specific requirement for free cholesterol which intercalates with caveolin-1(CAV-1). It has been demonstrated that cerivastatin down-regulated cyclin D1 and decreased the cell growth in ERα-positive breast cancer cells; MCF-7, and this antiproliferative effects may be mediated through a reduction of caveolin-1 expression (Salis *et al.*, 2014). This work further highlights the roles statin plays in ERα-positive breast cancer cells and can lead us to more promising therapeutics targeting cholesterol metabolism in breast cancer or by using statins in combination with agents targeting the ERα.

4.7. Conclusion

27-hydroxycholesterol plays a role in breast cancer progression. In this study, we found that 27OHC induced proliferation in the ERα-positive tumour cells, but not in ERα negative cell lines. Furthermore, 27OHC induced migration and invasion, in both ERα positive and negative cell lines. Our data indicates that whilst the proliferative effects of 27OHC were dependent on the presence of the ERα, its actions on migration and invasion were mediated by a different pathway with preliminary data suggesting a role for ERβ.

Chapter 5.

The involvement of the ERβ in the role of 27OHC on breast cancer cell migration/invasion and its regulation of the IGF-I and EGF receptors.

5.1 Introduction

The estrogen receptors (ERs), ERα and ERβ play a crucial role in hormone-dependent breast cancer development and metastasis, by mediating the effects of estrogen(Osborne *et al.*, 2001). ERs can also be regulated independently of estrogen, such as via activation of growth factor receptors (McDonnell and Norris, 2002). ERα expression was found to be 10% in normal breast epithelial cells and 50–80% in breast tumors (Huang, Warner and Gustafsson, 2015b).

While ERα is well recognized for contributing to breast cancer growth and development, the role of ERβ is less understood. In 1996, ERβ was discovered, and its function in breast cancer is still being elucidated (Haldosén, Zhao and Dahlman-Wright, 2014b). The activation of ERβ can form heterodimers and/or homodimers with the ERα (Paech *et al.*, 1997), thus modulating the activity of ERα. It has been demonstrated that the relative expression levels of ERβ and ERα are likely to influence cell growth and the activities of different signalling cascades in breast cancer cells (Chang *et al.*, 2006). The dysregulation of ER signalling is linked with progression and initiation of several cancers, such as breast cancer as it affects a number of different molecules, such as cyclin D1, insulin-like growth factor-1 receptor (IGF-IR), vascular endothelial growth factor (VEGF) and anti-apoptotic BCL-2 protein, that impact on cell proliferation, survival and growth (Umetani, 2016).

ERβ has five different isoforms, therefore these multiple isoforms complicate the investigation of the biological role of ERβ and its participation in the carcinogenesis of breast tumours (Girgert, Emons and Gründker, 2019). The five isoforms of ERβ (β1, β2, β3, β4, and β5), do not all bind to estrogen (Honma *et al.*, 2008; Zhang *et al.*, 2012).

Early studies proposed that ERβ is a risk factor linked with breast cancer (Haldosén, Zhao and Dahlman-Wright, 2014b). Furthermore, the estrogen receptor ERβ is found in 44.4% of TNBC (Matthews and Gustafsson, 2003). A recent studies indicate elevated levels of ERβ targets genes that regulate cell survival/death, cell movement, growth and cell development, along with genes linked with the Wnt/β-catenin and G1/S cell cycle phase checkpoint pathways (Shanle *et al.*, 2013; Hamilton *et al.*, 2015).

In recent years, 27OHC, an oxysterol has been identified as an agonist of ERα and an antagonist of ERβ (Herynk and Fuqua, 2007). The content of 27OHC in ERα-positive breast cancer cells is 2.5-3-fold higher than in normal breast cells (Wu *et al.*, 2013). 27OHC may modify cancer risk in different ways, such as modulation of signalling (e.g., Wnt, Hedgehog) and inflammatory pathways (Kloudova, Guengerich and Soucek, 2017). The proliferative role of 27OHC has been confirmed in human cancer cells (Nelson *et al.*, 2013a) and in human breast cancer tissue, 27OHC concentrations and the levels of the enzyme, CYP27A1 were increased in higher grade tumours (Baek *et al.*, 2017).

Emerging studies illustrates that metabolic factors, for instance insulin-like growth factor (IGF-I and IGF-II) signalling pathways promote the development of breast cancer (Belardi *et al.*, 2013; Sturtz *et al.*, 2014). ER signalling pathways are known to intersect with IGF signalling cascades, with estrogen increasing breast cancer production of IGF-II (Westley and May, 1994). Aberrant EGF and IGF signalling is detected in TNBC tumours and is linked to higher rates of recurrence, poor response to therapy and reduced overall survival. Experimental and clinical evidence implicates the EGF and IGF systems in the progression of breast cancer (Samani *et al.*, 2007; Voudouri Kallirroi *et al.*, 2015; Christopoulos, Corthay and Koutsilieris, 2018b).

5.2 Aims

In this chapter, we aimed to investigate the involvement of the ERβ in the effects of 27OHC on breast cancer cell migration/invasion, and its regulation of IGF and EGF receptors.

5.3 Specific aims

Aim 1: To optimise shRNA-mediated knockdown of ERβ in MDA-MB-231 TNBC cells.

Aim 2: To assess the role of the ERβ in the effects of 27OHC on cell migration and invasion.

Aim 3: To establish a role for the ERβ in the regulation of IGF-I and EGF receptors and associated signalling molecules

5.4. Methods

5.4.1. Cell culture:

The human breast cancer cell lines MCF7 and MDA-MB-231 were obtained from ATCC (Teddington, Middlesex, UK). All cell lines were cultured as described previously in section 2.1.1.

5.4.2. Western blot

Western blot analysis was observed as described previously (2.10). In brief, protein cell lysates (30µg), were allocated in SDS-PAGE gel, transferred to nitrocellulose membrane and immunoblotted with the targeted antibodies: anti-ERβ (1:1000) (PPZ0506: Thermo Fisher; 14C8: Abcam, and 05-824; Merck Millipore), IGF-I Receptor β (1:1000; Cell Signalling), EGF Receptor (1:1000; Cell Signalling), GAPDH (1:5,000; Millipore) and β-actin (1:10,000; Sigma-Aldrich). After that the membranes were incubated with particular secondary antibodies (anti Mouse or Rabbit) conjugated to peroxidase, next proteins were detected by Clarity ECL substrate and quantified using Image software

5.4.3. Optimizing an ERβ antibody

Antibody validation is critical to generate accurate valid data as demonstrated in a recently published report where previous studies on ERβ were disputed (Andersson *et al.*, 2017). ERβ antibodies for Western blotting were first validated by screening 3 antibodies, including the most commonly used ones (14C8; Abcam, PPZ0506; Thermo Fisher and 05-824; Merck Millipore). Western blotting was used to assess the protein abundance of ERβ in different breast cancer cell lines. The PPZ0506 ERβ antibody displayed a single band of the expected size (the molecular weight; 59 kDa) in lysates from a panel of breast cancer cell lines (Fig. 5.3A), however the Merck Millipore ERβ antibody displayed a single band

below the expected size (57 kDa) (Fig. 5.3B). Using the Abcam ERβ antibody, multiple bands including a weak band at the correct size were seen (Fig. 5.1C). ERβ protein levels seemed to be higher in the normal like breast cancer cell line (MCF10A) and ERα-negative (MDA-MB-231) compared with ERα+ve (MCF-7 and T47D), as shown in (Fig. 5.1 A). We concluded that ERβ (PPZ0506; Thermo Fisher), is the most appropriate antibody to use for all further experiments.

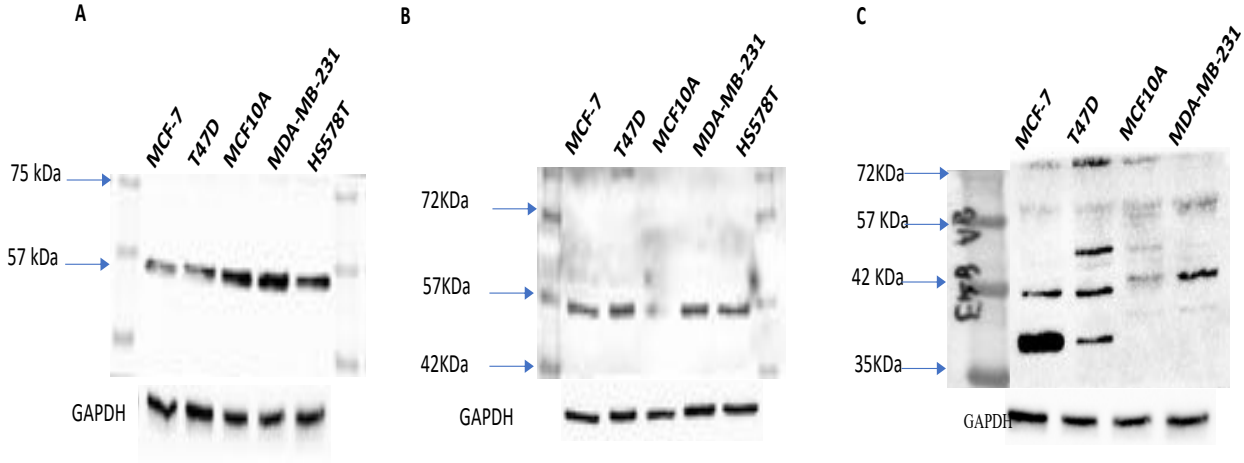


Figure 5. 1: ERβ antibody assessment using a panel of breast cancer cell lines: *Representative images of western blotting: A) ERβ (PPZ0506; Thermo Fisher), B) 05-824; Merck Millipore, and C) 14C8; Abcam antibody in normal breast epithelial cell line, MCF-10A, and 4 cancerous cell lines – ERα-positive (MCF-7 & T47D) and ERα-negative (MDA-MB-231 and Hs578T). GAPDH was used as loading control protein. Data representative of (n=3).*

5.4.4. RNA extraction and RNA-cDNA reverse transcription

Trizol reagent (Invitrogen) was used to extract RNA as outlined in section (2.11.1).

5.4.5. Quantitative Polymerase Chain Reaction (qPCR)

Quantitation of expression of ERβ mRNA was performed using SYBR green- based qPCR. The StepOnePlus was used to analyze the PCR reaction and relative mRNA levels were

assessed using the $2^{-\Delta Ct}$ method after normalization to the GAPDH reference gene as described previously in section (2.11.4)

5.4.6. Dosing with exogenous 27OHC, LDL and ERβ-agonist and antagonist:

For proliferation assays, cells were incubated in growth media DMEM for 24 hours, before being changed to serum-free media for a further 24 hours. The cells were then treated with different concentrations of LDL and 27OHC and as described in chapter 3 and 4 respectively. To investigate the optimum concentration of a selective ERβ antagonist, 4-[2-phenyl-5,7- bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol (PHTPP), MDA-MB-231 and MCF-7 cells were treated with different concentrations ranging from 0-5μM. Effective treatment of PHTPP was shown by a reduction in levels of ERβ protein by Western blotting. As shown in Figure 5.2A the levels of ERβ decreased gradually with increasing concentration of PHTPP compared with control in MCF-7 cells, with levels reduced only at 5μM in MDA-MB-231 cells (Fig. 5.2B).

Figure 5. 2: The optimum concentration of ERβ antagonists in MCF-7 and MDA-MB-231 cell lines:

Cells were treated with increasing doses of a selective ERβ antagonist (PHTPP)(0-5μM). Western blotting was used to detect the abundance of ERβ with or without PHTPP in A) MCF-7 and B) MDA-MB-231. B-actin was used as loading control. Data representative of (n=1).

To further investigate the role of ERβ, we activated ERβ in MDA-MB-231 cells using Erteberel (LY500307) that has selective binding affinity for ERβ. We treated MDA-MB-231 cells with different concentrations of Erteberel ranging from 0-10μM for 24 hours. Effective concentrations of the ERβ were assessed by qPCR (Fig. 5.3). The expression of ERβ increased in comparison to control with a peak response observed at 2.5μM in MCF-7 (Fig. 5.3A) and MDA-MB-231 (Fig. 5.3B).

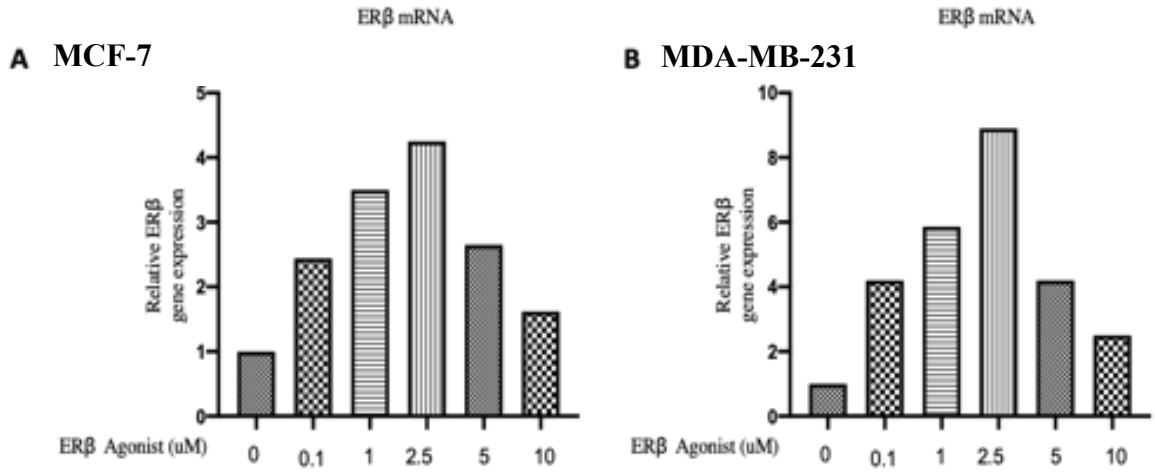


Figure 5. 3: Assessment of ERβ mRNA expression using quantitative polymerase chain reaction (qPCR):
Cells were treated with different concentration of a selective ERβ agonist (0-10μM) for 24 hours. The mRNA level of ERβ was quantified using qPCR in A) MCF-7 and B) MDA-MB-231. Data representative of (n=1).

5.4.7. Cell proliferation

This was determined using a CV proliferation assay (Feoktistova, Geserick and Leverkus, 2016b), as described in section 2.6.

5.4.8. Targeted ERβ gene knockdown:

5.4.8.1. siRNA knockdown

For experiments investigating the effect of 27OHC on the cell migration of MDA-MB-231 cells in presence or absence of ERβ, the optimum concentration of siRNA for silencing the Erβ (Dharmacon) or non-silencing (NS) (AllStars) was investigated. MDA-MB-231 cells were transfected with different concentration of siRNA ranging from 0-60nM or non-silencing (NS) (AllStars). Effective silencing of the ERβ was shown by Western blotting (Fig. 5.4 A) and qPCR (Fig. 5.4 B). Figure 5.4 A and B show effective silencing of ERβ at the concentration of 40nM compared with non-silencing siRNA

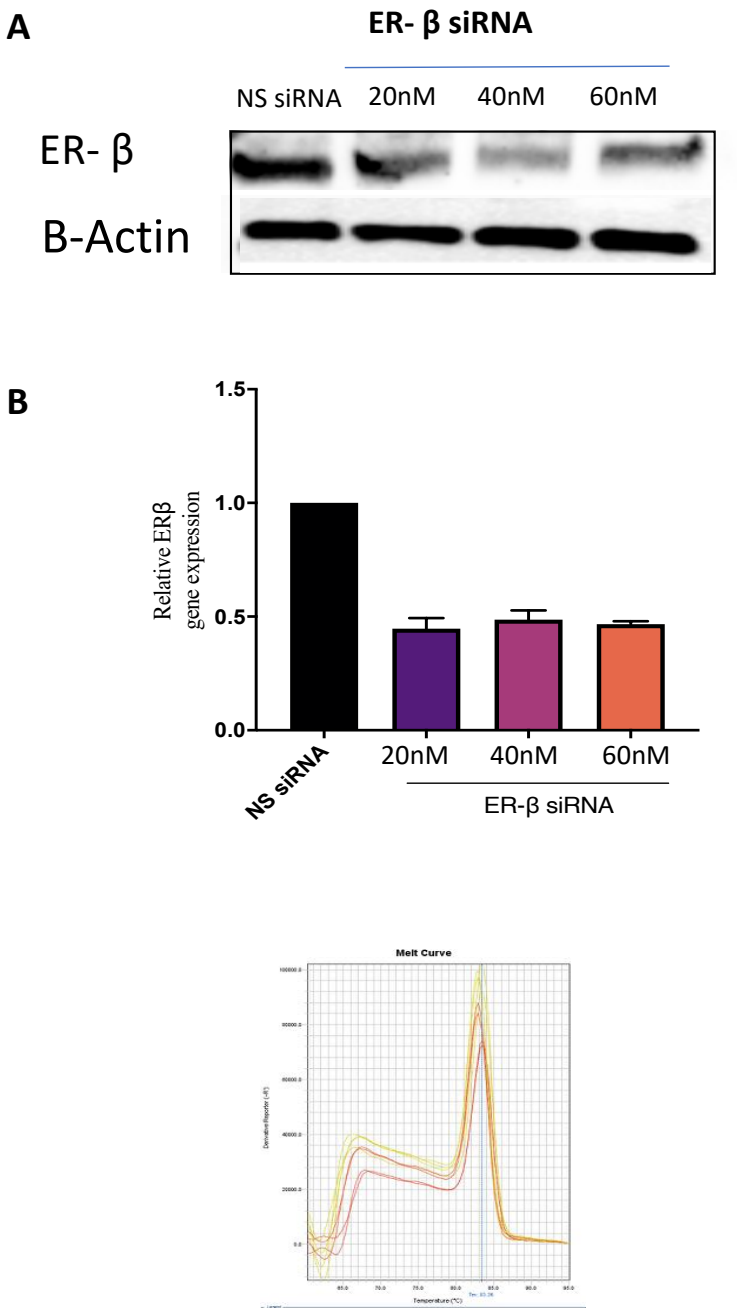


Figure 5. 4: Optimizing ERβ siRNA in MDA-MB-231 cell line:
MDA-MB-231 after being transfected with ERβ siRNA (20,40 and 60nM) and non-silencing RNA. A) Representative images of western blotting assess the protein abundance of ERβ expression. B-actin was used as loading control protein. B) mRNA expression of ERβ and melt curve of ERβ. GAPDH was used as a house keeping gene. Data representative of (n=1).

5.4.8.1. Development of stable knockdown of ERβ using shRNA in MDA-MB-231 cell line:

Transduction with lentiviral vectors is a highly efficient method to modulate expression of target genes (Blömer *et al.*, 1997).

To further understand the role of ERβ (ESR2) in TNBC, a stable transfection model for knockdown of ERβ was developed for further work. To perform the stable knock down, different concentrations of puromycin dihydrochloride, which kills cells that do not express resistance genes, ranging from 0.125–1 μg/mL were used for 7 days. The kill curve shows the optimal working concentration to be 0.8 μg/mL, which is the lowest puromycin concentration at which all available cells are dead after 7 days of puromycin selection (Fig. 5.5A &B).

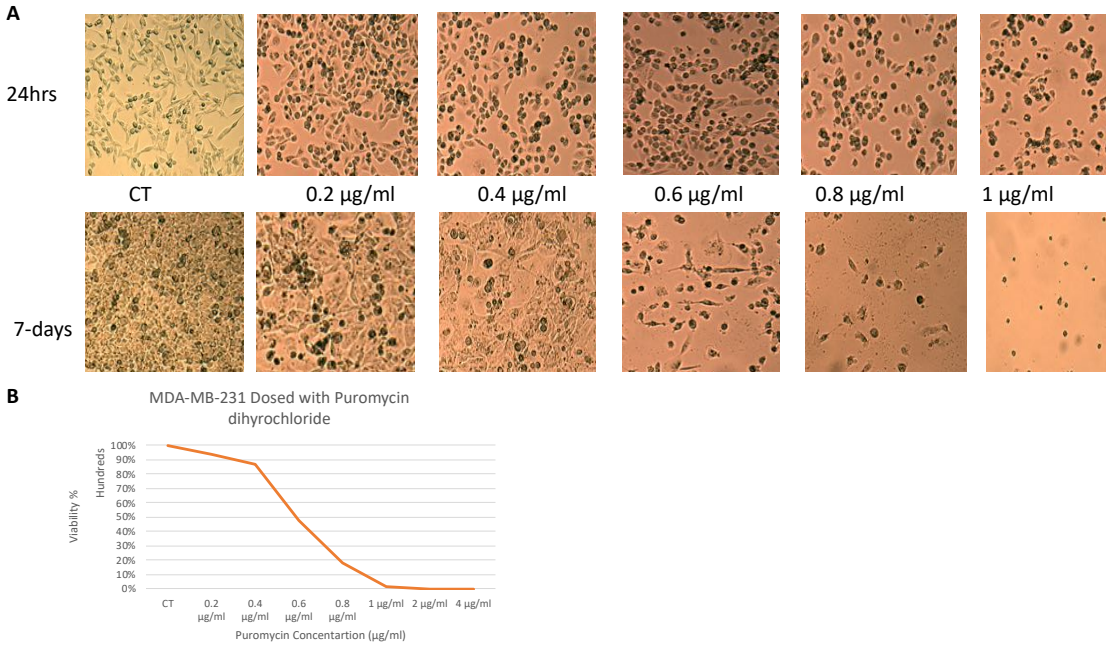


Figure 5. 5: Assessment of antibiotic, puromycin, kill curve for MDA-MB-231:

Cells were treated with increasing concentrations of puromycin (0.8, 0.4,0.60.8 & 1µg/ml) for168hrs. A) Representative images of MDA-MB-231 cells treated with puromycin after 24hrs and 168hrs time points. B) Cell viability using Trypan blue staining, shows the puromycin kill curve from 0hrs-168hrs. Data representative of (n=1).

Following several cell passages after transfection with two different amounts (shRNA1and shRNA2; 10 and 15µl/well respectively following the manufacture instruction) of ,1x 10^6 infectious units of virus (IFU), shRNA against ERβ: shRNA target-specific human ERβ or non-targeting shRNA control, as described in section 2.8, a gradual decrease in ERβ expression was observed. The transfected cells with ERβ shRNA were followed by a final reduction in the ERβ protein expression at the level of 80.09% ($p < 0.01$) for and ERβ shRNA2, in comparison with the control shRNA2, lentiviral particles, transfected cells and empty control. Stable transfection of ERβ shRNA2 resulted in a 65.20% decrease of ERβ

gene expression in comparison with the control shRNA (Fig. 5.6 A-B). This suppression was examined after four cell passages and remained constant in following cell passages.

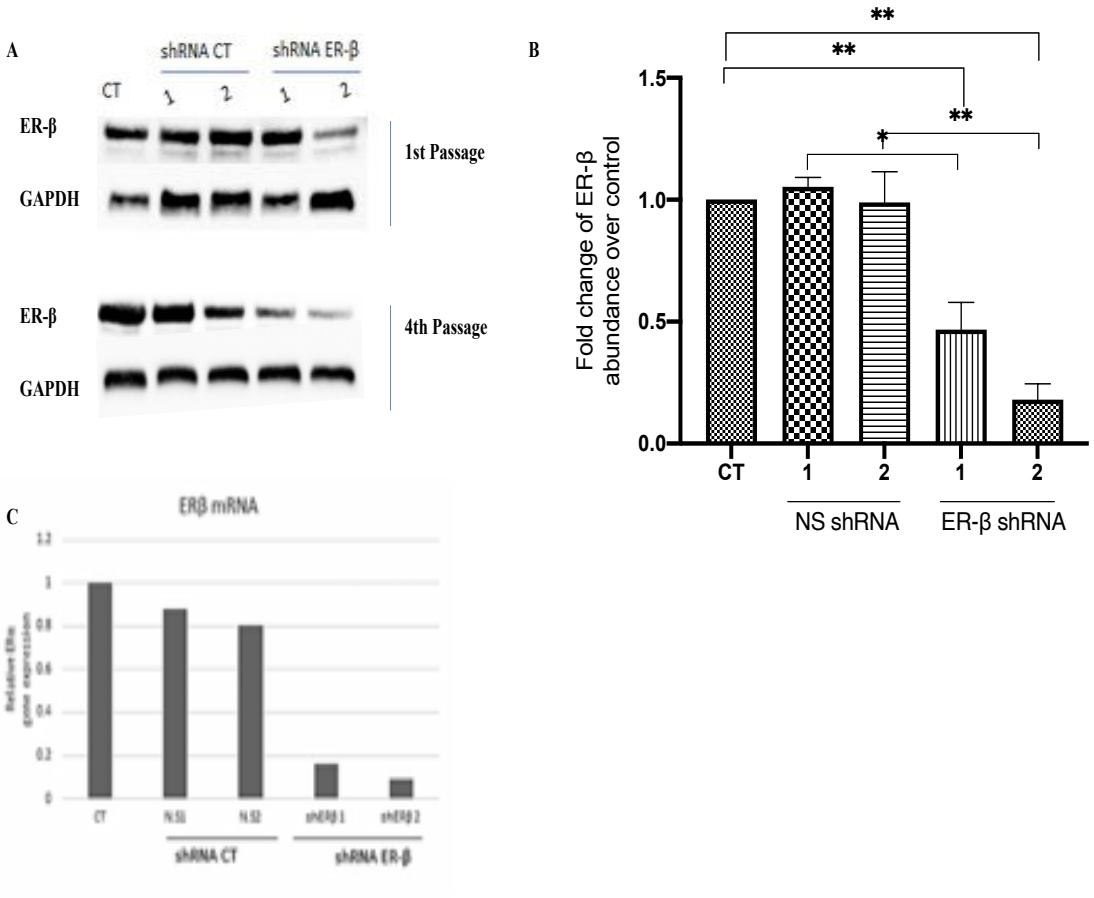


Figure 5. 6: ERβ Knockdown in MDA-MB-231 breast cancer cells:

*Cells were stably transfected with two different concentrations (shRNA1;5µl/well and shRNA2;15µl/well) of shRNA against ERβ or control lentiviral particles or negative control (empty). A) Western blotting was used to detect the abundance of ERβ with or without ERβ knockdown with two different concentration of ERβ shRNA (1&2) after the first and fourth passages. B) Densitometry quantification of ERβ was assessed after normalization to GAPDH. C) mRNA expression of ERβ, GAPDH was used as a house keeping gene. Data representative of mean ±SEM (n=3) except C) (N=1). P-value were determined by using GraphPad Prism: one-way Statistical analysis: one-way ANOVA test flowed by Tukey's post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

5.4.9. Transwell migration assay

Cells were prepared to migrate into the chemoattractant serum in the lower compartment as described in section 2.9, for 24 h (MCF-7) or 6 h (MDA-MB-231) with or without the addition of 27OHC (0.1μM) or ERβ siRNA/shRNA.

5.4.10. Statistical analysis

The whole experiments were repeated in triplicate, and also each experiment was repeated three times. Using GraphPad Prism 8.0.1 software for windows (La Jolla, CA, USA), to analyse the data, one-way ANOVA following the least significant difference (LSD) post-hoc test.

5.5. Results

5.5.1. Is the effect of 27-hydroxycholesterol on the migration and invasion of MDA-MB-231 breast cancer cells mediated by ERβ?

ERβ protein levels were higher in the normal-like breast cancer cell line (MCF10A) and ERα-negative (MDA-MB-231) cells compared with ERα-positive (MCF-7 and T47D) cells, as shown in the methods (Fig. 5.1A).

To assess whether the ERβ played a role in the actions of 27OHC on breast cancer cell migration and invasion, MDA-MB-231 cells were selected as these possess abundant ERβ but lack ERα as shown in figure 5.7. To understand the role of ERβ, we inhibited ERβ in MDA-MB-231, using three different strategies (siRNA, shRNA or an ERβ inhibitor, PHTPP).

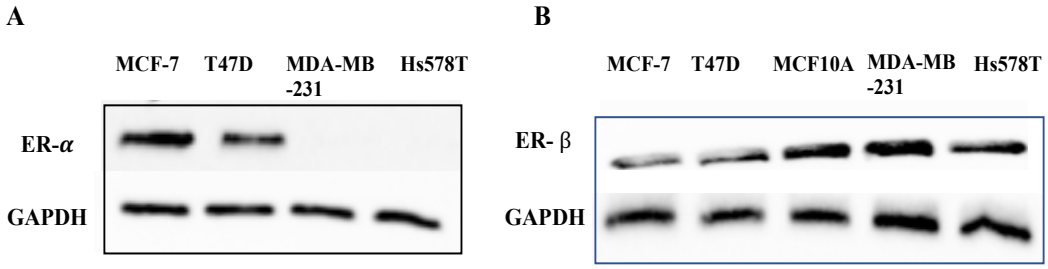


Figure 5. 7: ERα antibody assessment using a panel of breast cancer cell lines: *Representative images of western blotting: A) ERα antibody in 4 cancerous cell lines – ERα-positive (MCF-7 & T47D) and ERα-negative (MDA-MB-231 and Hs578T). and B) ERβ antibody in 4 cancerous cell lines – ERα-positive (MCF-7 & T47D), ERα-negative (MDA-MB-231 and Hs578T) and a normal epithelial breast cell line (MCF10A). GAPDH was used as loading control protein. Data representative of (n=1).*

The results showed that 27OHC (0.1 μ M) increased cell migration ($p < 0.001$) and invasion ($p < 0.001$) and these effects were blocked with ERβ silenced using siRNA (Fig.5.8A, B & C respectively). These observations were confirmed using a selective ERβ antagonist,

PHTPP: where 27OHC was similarly unable to increase migration in the presence of PHTPP (Fig. 5.8 D). Interestingly, using the publicly available METABRIC gene expression database, it was observed that the breast cancer patients who had high mRNA levels of CYP27A1 (and thus expected elevated 27OHC) also had increased expression of ERβ mRNA levels ($p<0.001$) when compared to normal breast tissue (Fig.5.8E). Conversely low tumour expression of CYP27A1 was associated with lower expression of ERβ.

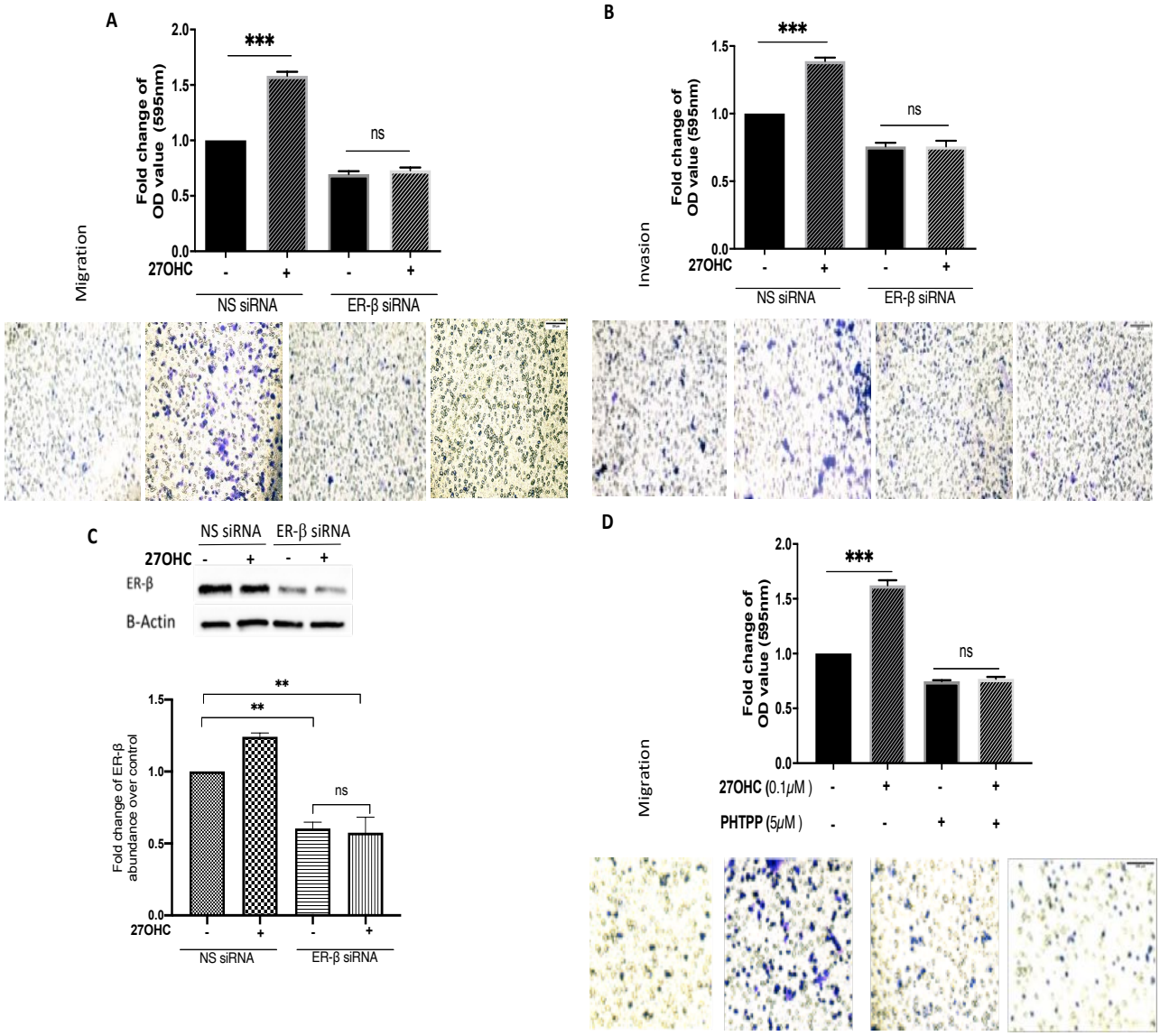


Figure 5. 8a: 27OHC increases cell migration/invasion in ERα-negative breast cancer cells via ERβ:

*A trans-well assay was used to detect A) migration and B) invasion after 6 hours for MDA-MB-231 after being transfected with ERβ siRNA (40nM) and non-silencing RNA (40nM) and dosed with 27OHC (0.1 μM). C) Western blotting was used to detect the abundance of ERβ with or without ERβ silencing. Densitometry quantification of the ERβ was assessed after normalization to β- actin. D) Quantification of cell migration after being dosed with 27OHC (0.1 μM) and PHTPP (5 μM). The migrated and invaded cells were stained with crystal violet and images were taken (×20 magnification). Results are presented as mean +/− SEM (n=3) (A-D). P-value were determined by using GraphPad Prism: one-way ANOVA followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001) (A-D). Scale bar represents 100μm.*

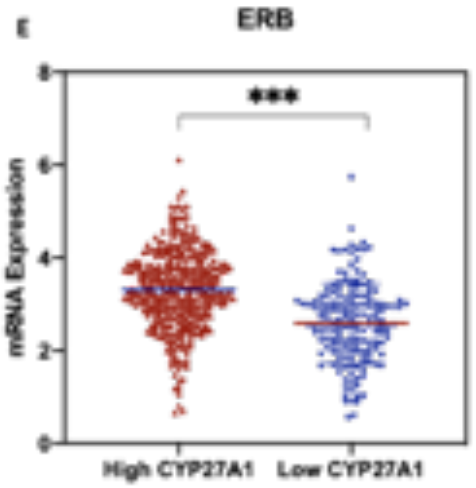


Figure 5. 9b: 27OHC increases cell migration/invasion in ERα-negative breast cancer cells via ERβ:
E) Scatterplot analysis of TCGA data of 504 invasive breast cancer carcinomas for mRNA expression of ERβ expressed as median expression of CYP27A1, where the horizontal line presents the mean. P-value was determined using GraphPad Prism: Mann–Whitney test.

We confirmed these observations using an shRNA mediated ERβ knockdown in the MDA-MB-231 cells. 27OHC was similarly unable to increase migration in the absence of ERβ (Fig. 5.9A). Knockdown of ERβ using shRNA constructs was confirmed by Western blot analysis (Fig. 5. 9B).

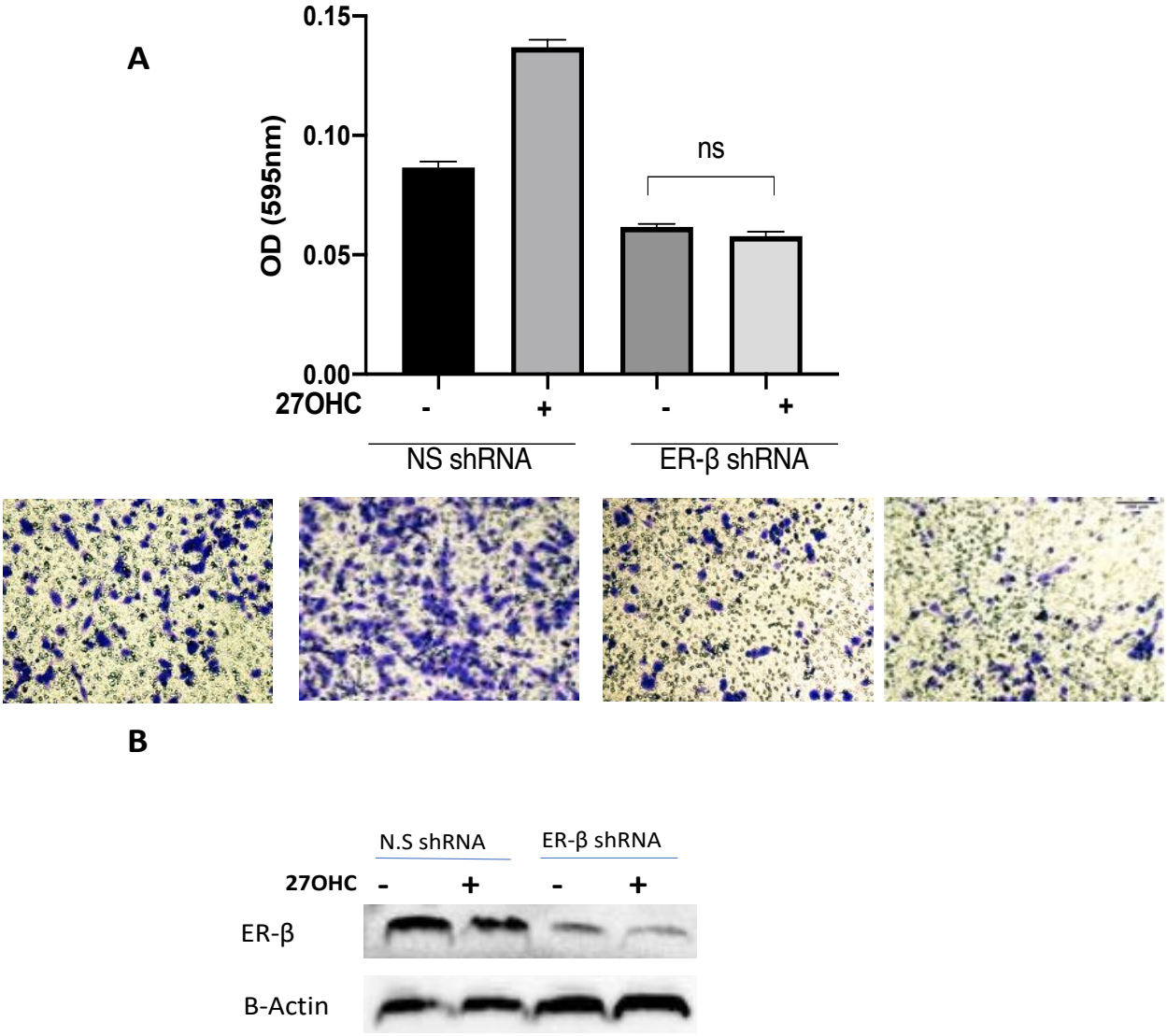


Figure 5. 10: 27OHC increases cell migration in ERα-negative breast cancer cells via ERβ:

MDA-MB-231 cell were stably transfected with shRNA for knockdown (ERβ) or control shRNA particles and dosed with 27OHC (0.1μM) for 48hrs. A) A trans-well assay was used to detect migration after 6 hours. The migrated and invaded cells was stained with crystal violet and images were taken (×20 magnification). B) Western blotting was used to detect the abundance of ERβ with or without ERβ silencing. B-actin was used as loading control. Data representative of (n=1). Scale bar represents 100μm.

5.5.2. The role of ERβ in the regulation of IGF-I and EGF receptors and associated signalling molecules.

Based on the wealth of pre-clinical data suggesting a role for the EGF and IGF signaling pathways that are linked to ER signalling in breast cancer, we further investigated the role of ERβ in the regulation of IGF-I and EGF receptors. The activation of ERβ significantly increased the abundance of the IGF-I and EGF receptors (Fig. 5.10). As shown in figure 5.10A and B, treatment with a synthetic ERβ agonist gradually increased levels of the IGF-I receptor level from 1μM in comparison to control with a peak response observed at 2.5μM; the percentage increases were respectively; (1μM 20.5%; ns), (2.5μM 120.7%; $p < 0.01$) in MCF7. A similar result was seen in MDA-MB-231 cells with a peak increase in IGF-I receptor level observed at 2.5μM ($p < 0.001$), and EGF receptor level observed at 2.5μM ($p < 0.05$) (Fig. 5. 10C & D).

Conversely, with all approaches of ERβ inhibition, we observed a loss of the IGF-I and EGF receptors (Fig. 5.10). With MCF-7 cells, we found that an ERβ inhibitor, PHTPP, significantly decreased the level of IGF-I receptor abundance with both concentrations at 5 and 10 μM ($p < 0.001$ and $p < 0.001$ respectively) (Fig. 5. 10A&B). We also found the same results with MDA-MB-231 cells, with a reduction in the IGF-I receptor levels observed at 5 and 10μM ($p < 0.01$ and $p < 0.01$ respectively), and with EGF receptor levels observed at 10mM ($p < 0.001$) (Fig. 5. 10E & F). Further, the ERβ inhibitor, PHTPP also caused an inhibition in cell proliferation in MDA-MB-231 cells; from 2μM in comparison to control with a further decline in response observed at 10μM; the percentage decreases

were (2mM 12.7%; $p < 0.001$), (5mM 22.0%; $p < 0.001$) and (10mM 29.73%; $p < 0.001$) in MDA-MB-231 cells (Fig. 5.11A). Interestingly, using the publicly available METABRIC gene expression database, we found that tumours that had high mRNA levels of ERβ also had increased expression of the IGF-IR and EGFR and Ki-67, as a marker of proliferating cells ($p < 0.001$, $p < 0.001$ and $p < 0.01$ respectively) (Fig. 5.11 B-D respectively). Conversely low tumour expression of ERβ was associated with lower expression of the IGF-IR and EGFR and Ki-67.

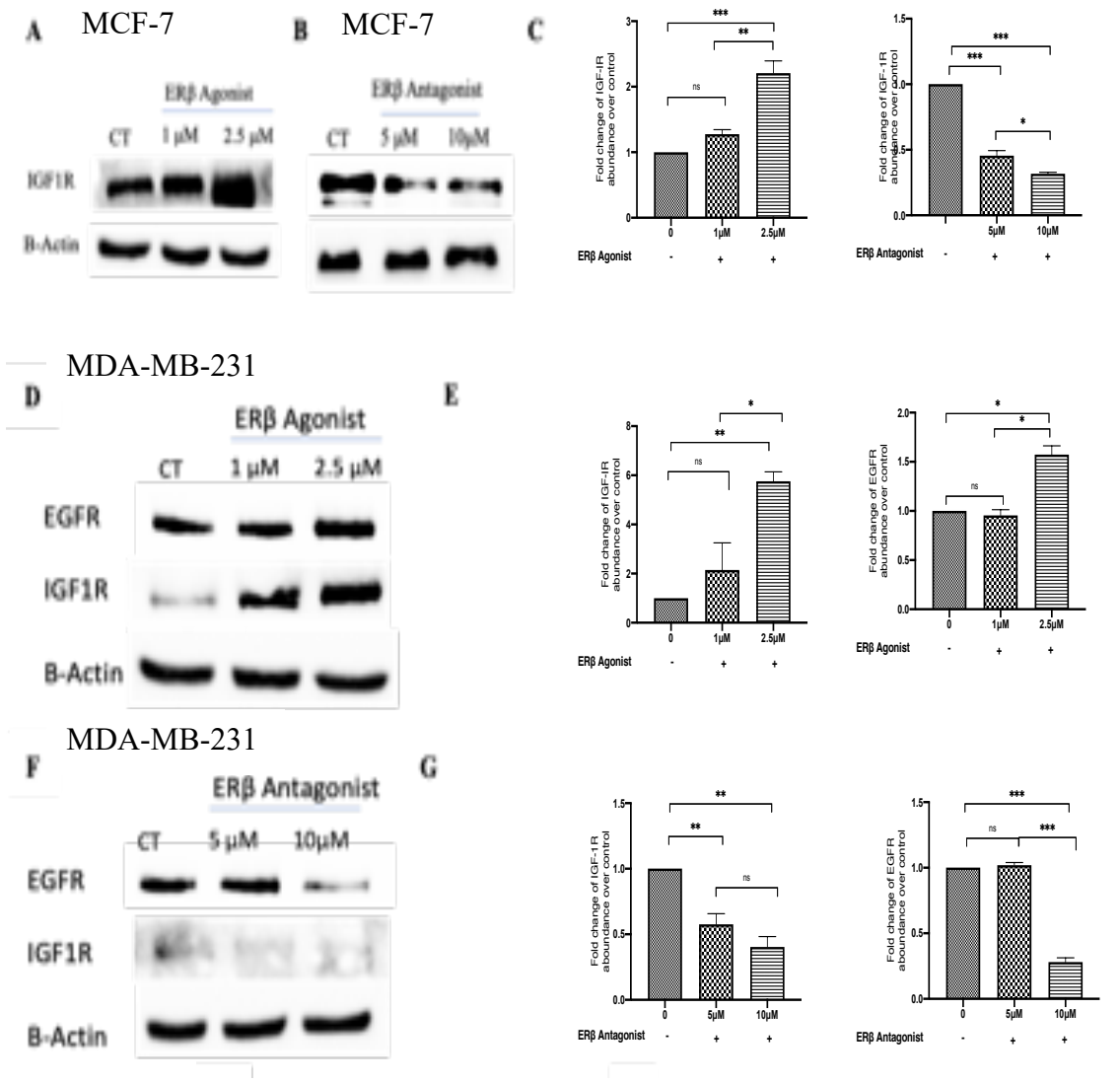


Figure 5. 11: Effects of selective ERβ agonist and antagonist treatment on IGF-IR and EGFR levels in MCF-7 and MDA-MB-231:

*(A-C) MCF-7 and (D-F) MDA-MB-231 treated with ERβ agonist for 24hrs and antagonist for 48hrs and were immunoblotted with IGF-IR (A-F) and (D-G) EGFR antibody. GAPDH worked as loading control. (C, E&G) Quantitative densitometry analysis. (H) MDA-MB-231 cells were treated with increasing doses of a selective ERβ antagonist for 48hrs and cell proliferation was assessed using a crystal violet assay. (I-K) Scatterplot analysis of TCGA data of 504 invasive breast cancer carcinomas for mRNA expression of IGF-I, EGFR and Ki67 represented as median expression of ERβ. Results are presented as mean +/− SEM (n=3) (A-D), P-value were determined by using GraphPad Prism: one-way Statistical analysis: one-way ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001) (A-G).*

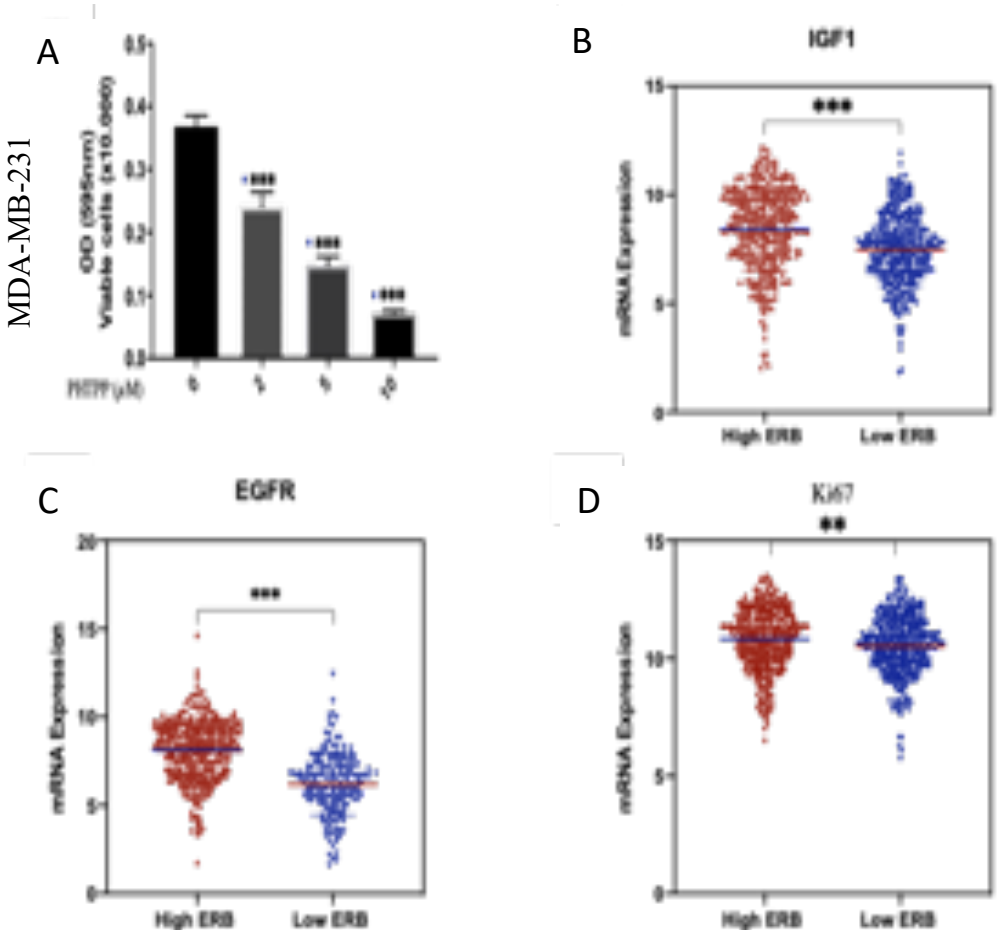


Figure 5. 11: The role of ERβ in the regulation of IGF-I, EGF receptors, Ki67 and cell proliferation.:

*(A) MDA-MB-231 cells were treated with increasing doses of a selective ERβ antagonist for 48hrs and cell proliferation was assessed using a crystal violet assay. (B-D) Scatterplot analysis of TCGA data of 504 invasive breast cancer carcinomas for mRNA expression of IGF-I, EGFR and Ki67 represented as median expression of ERβ. Results are presented as mean +/− SEM (n=3) (A), P-value were determined by using GraphPad Prism: one-way Statistical analysis: one-way ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001) (A), B-D) Mann–Whitney test.*

We also observed a loss of the IGF-I receptor with ERβ siRNA in MCF-7 (Fig. 5.11A), the IGF-I and EGF receptors with ERβ siRNA and shRNA in MDA-MB-231 (Fig. 5.11B &C respectively).

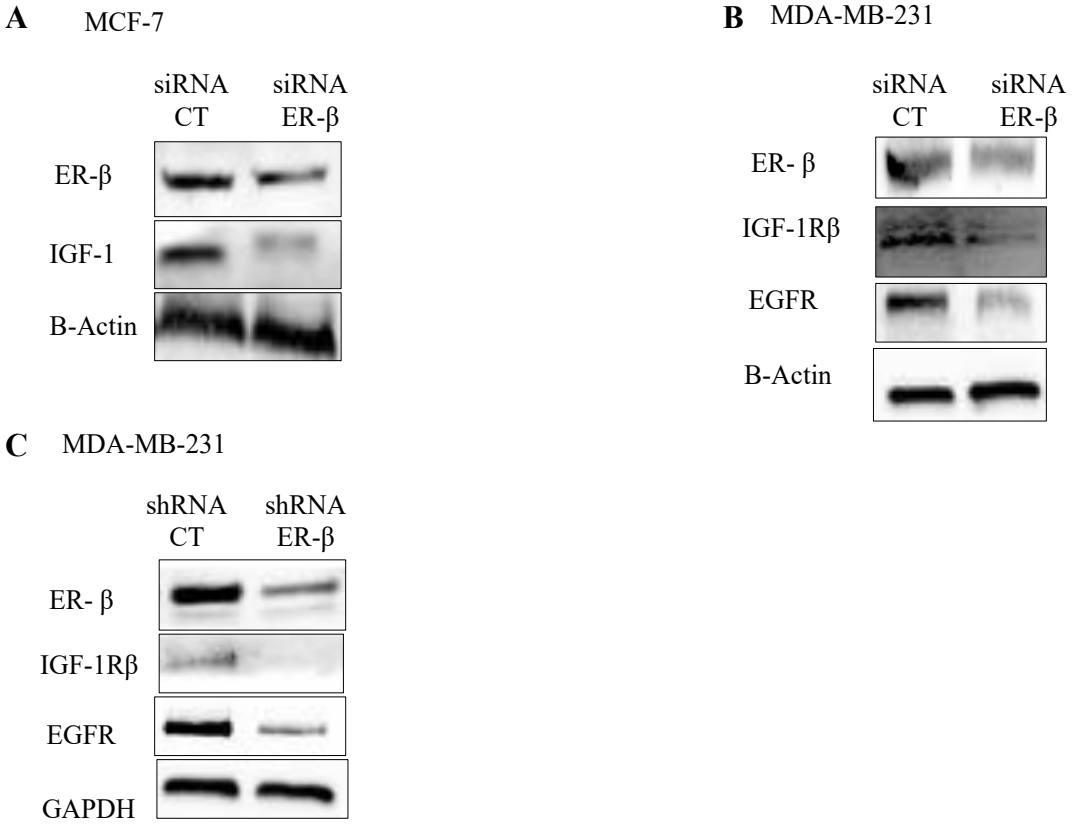


Figure 5. 12: Effects of knocking down ERβ on levels of the IGF-IR in MCF-7 and of the IGF-IR & EGFRs in MDA-MB-231:

MCF-7 (A) and MDA-MB-231 (B) after being transfected with ERβ siRNA and non-silencing RNA for 48hrs, (C) MDA-MB-231 after being transfected with ERβ shRNA and non-silencing shRNA and were immunoblotted with IGF-IR, ERβ (A,B&C) and EGFR (B&C) antibodies. GAPDH was used as a loading control. Data representative of mean ±SEM (n=2).

5.5.3. The involvement of the 27OHC in regulating molecules associated with IGF signalling pathway,

Caveolae have a particular requirement for free cholesterol which intercalates with caveolin-1 (CAV-1) (Murata *et al.*, 1995). CAV-1 has been shown to bind to the EGFR and IGF-1R and mediate their signaling. In our laboratory, we showed previously that CAV1 is a marker of an invasive, hormone-resistant phenotype and correlates with the beta1 integrin in TNBC cells (Zielinska *et al.*, 2018). As we observed that the addition of LDL increased the abundance of key cell surface markers including the IGF-1R, we wished to determine if CAV1, and beta 1, which we have shown play important roles, were also involved. We examined the effect of 27OHC on the abundance of CAV1 (a marker of lipid rafts), the β1 integrin (that associates with CAV1) and fibronectin. The addition of 27OHC for 48hrs significantly increased the abundance of CAV-1, fibronectin and the β1 integrin ($p < 0.05$, $p < 0.05$ and $p<0.05$ respectively) (Fig. 5.12A &B and Fig. 5.13). Figure 5.13 shows that the 27OHC-induced increase in the abundance of the fibronectin was blocked in the absence of ERβ.

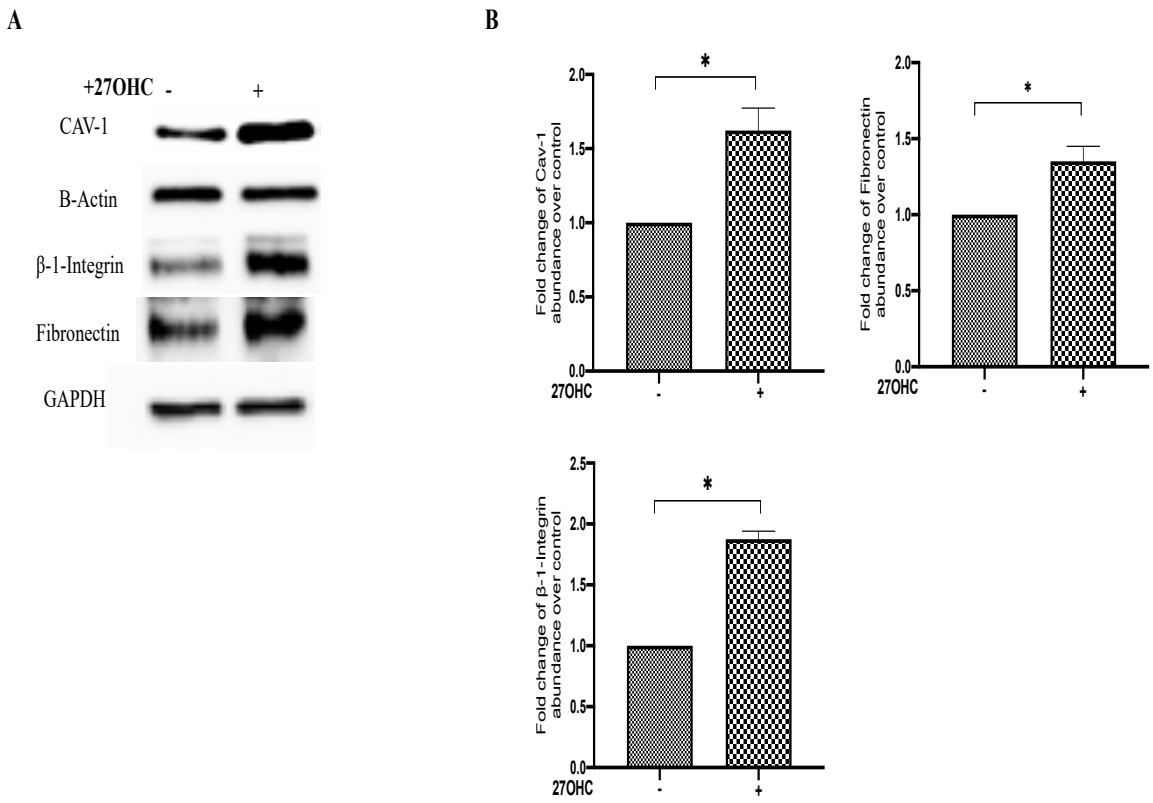


Figure 5. 13: The involvement of the 27OHC in regulating key signalling molecules in lipid rafts:

(A*) MDA-MB-231 were treated with 27OHC for 48hrs and were immunoblotted with indicated antibodies. GAPDH and B-actin were used as loading controls. (B) Quantitative densitometry analysis; the data are expressed as mean ± SEM (n=3), and P-value were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

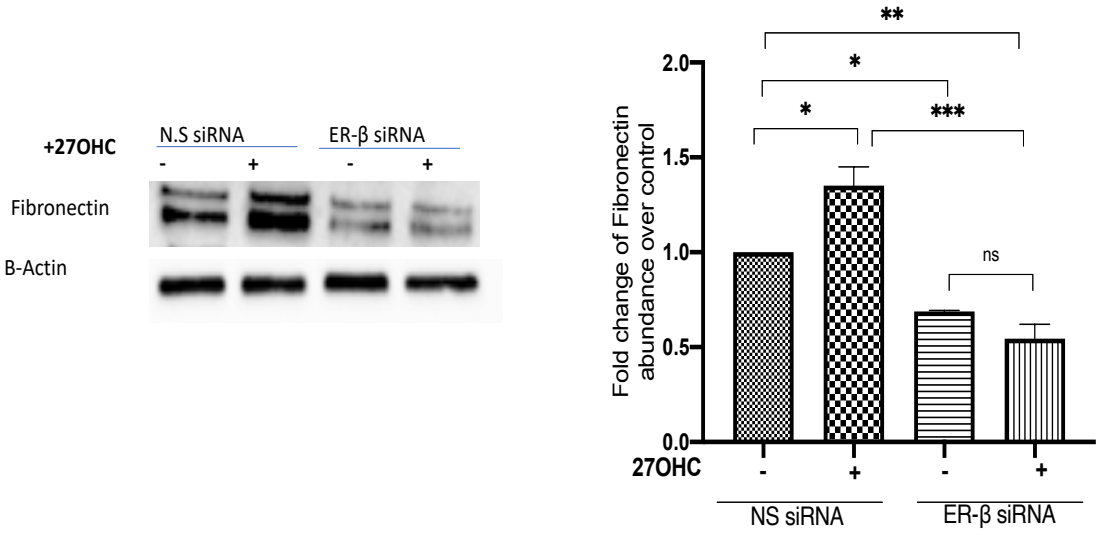


Figure 5. 14: The effect of the ERβ on fibronectin levels in the present or absence of 27OHC:

*MDA-MB-231 after being transfected with ERβ siRNA and non-silencing RNA treated with 27OHC for 48hrs were immunoblotted with indicated antibodies. B-actin worked as loading control. Quantitative densitometry analysis: the data are expressed as mean ± SEM (n=3), and P-value were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test followed by least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

5.6. Discussion

In the previous chapter, 27OHC was shown to promote cell growth in ERα-positive breast cancer cells and increased breast cancer cell migration and invasion in both ERα and β positive cell lines. When silencing the ERα in MCF-7, we found that only the effect of 27OHC on cell proliferation was inhibited and not its ability to increase cell migration and invasion. Our data also suggested that the only receptor increased when silencing ERα in ERα-positive cell lines, was ERβ. This corresponds to previous reports whereby the role of ERβ depends on the relative ratio of expression of ERα to ERβ. ERβ having a protective effect in the presence of ERα but in the absence of ERα recent data suggest that ERβ has a pro-tumourigenic role (Leygue and Murphy, 2013). Approximately 30% of TNBCs express ERβ. It has been demonstrated that ERβ target genes can regulate cancer cell survival/death, movement, growth and development as well as genes associated with G1/S cell cycle phase checkpoint pathways and the Wnt/β-catenin and these are elevated in TNBC (Shanle *et al.*, 2013; Hamilton *et al.*, 2015). Higher expression level of ERβ in TNBC makes it a logical pharmacological target due to its structural similarity to ERα (Novelli *et al.*, 2008b). However, there have been conflicting results from studies of the role of ERβ in TNBC because of the fact that TNBC is a heterogeneous disease, and this is not reflected in *in vitro* studies when using TNBC cell lines (MDA-MB-231 and Hs578T). Furthermore, discrepancies in the reported results may also be because of a lack of standardized observation strategies, such as use of antibodies that have not been correctly validated, inconsistent cut-offs for specifying ERβ-positive cancers when using immunohistochemistry, and variable tissue processing and preparation methods (Andersson *et al.*, 2017). To address these inconsistencies we used a certified TNBC cell

line (negative for ERα. PR, and HER2 overexpression) and validated ERβ antibodies (Andersson *et al.*, 2017).

We observed that 27OHC drives the invasion and migration of MDA-MB-231 TNBC cells. Whilst a recent report proposed that 27OHC acted as a negative modulator of ERβ (Starkey *et al.*, 2018), we found effects of 27OHC in MDA-MB-231 cells were blocked by inhibiting ERβ using siRNA. 27OHC has both agonistic and antagonistic effects on ERα function depending upon on the target organs (Umetani *et al.*, 2014). Therefore, it is also possible that 27OHC may also have both agonistic and antagonistic effects on ERβ function. Our data showing a positive correlation between 27OHC and ERβ is consistent with the results from the TCGA database: we found that tumours that had high mRNA levels of CYP27A1 (and thus expected elevated 27OHC) also had increased expression of ERβ.

To further understand the role of ERβ we inhibited ERβ in MDA-MB-231 TNBC cells using three different strategies (siRNA, shRNA or an ERβ inhibitor, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol, PHTPP). With all approaches of ERβ inhibition, we observed a loss of the IGF-I and EGF receptors and an inhibition in basal cell proliferation. The phenotype that we observed has been confirmed recently by others (Piperigkou *et al.*, 2016). These data indicate that ERβ is a positive regulator of the IGF-I and EGF receptors. A link between ERβ and IGF and EGF signalling pathways was also indicated by silencing ERβ in TNBC cell lines, that reduced cell proliferation, migration and invasion that was associated with down-regulation of the IGF-I and EGF receptors. Conversely up-regulating ERβ, using an agonist, increased levels of the IGF-IR (Richardson *et al.*, 2011; Hamilton *et al.*, 2015). Some studies have indicated that ERβ found to be a biomarker linked with a more aggressive clinical outcomes (Novelli *et al.*,

2008a), and relates to Ki-67, a proliferation marker. This is consistent with the results from the TCGA database, in which we found that tumours that had high mRNA levels of ERβ also had increased expression of the IGF-IR, EGFR and Ki-67, mRNA levels.

The addition of 27-OHC for 48hrs increased the abundance of CAV1 and the β1 integrin. Depleting or sequestering cholesterol from the cell membrane leads to a shift in lipid raft and hormonal receptors and caveolar proteins such as CAV-1 to other parts of the plasma membrane. In our laboratory, Zielinska *et al.* have illustrated that CAV1 is a marker of an invasive, hormone-resistant phenotype (Zielinska *et al.*, 2018) and that CAV-1 associates with the β1 integrin in TNBC cells (Burrows *et al.*, 2006). We found that the addition of 27OHC increased the abundance of fibronectin and this was blocked in the absence of ERβ. This is also in agreement with a previous study indicating that ERβ suppression decreases the expression of the mesenchymal markers vimentin and fibronectin and decreases MDA-MB-231 breast cancer cells migration and invasion (Piperigkou *et al.*, 2016).

Based on the wealth of pre-clinical data suggesting a role for the IGF signalling pathways in TNBC, different strategies were developed to target these systems. Monoclonal antibodies and small molecules, targeting the tyrosine kinase domain of the receptors (TKIs) have been major approaches to disrupt IGF signalling cascades (Christopoulos, Corthay and Koutsilieris, 2018a) and these strategies applied as monotherapies have had limited clinical success. We elucidated that the phenotypic effects of 27OHC involved the IGF-IR and that perhaps targeting ERβ in TNBC could be a different approach for down-regulating IGF signalling.

Statins also impact on the pathways activated by IGF and EGF. Simvastatin has been shown to inhibit the development of radioresistant esophageal cancer cells by reversing the

process of EMT via the PTEN-PI3K/AKT signalling cascades and this action was blocked by the addition of IGF-I (Li *et al.*, 2014). This work further highlights the roles statins plays in aggressive breast cancer and can lead us to more promising therapeutics targeting cholesterol metabolism in TNBC or by using statins in combination agents targeting the ERβ.

5.7. Conclusion

In summary, our data elaborates a mechanism to support the clinical studies suggesting the link between obesity and high cholesterol with an increased risk of breast cancer progression: we identified a novel mechanism whereby 27OHC promotes cell migration and invasion through ERβ in TNBC cells. Our data also indicate that ERβ is a positive regulator of the IGF-I and EGF receptors. We observed that ERβ depletion decreased CAV-1, β1 integrin and fibronectin (a marker of EMT) in MDA-MB-231 TNBCs.

Chapter 6.

The effects of interactions between IGFs and cholesterol on breast cancer cell growth and migration

6.1. Introduction

Obesity is correlated with an increase in cancer-specific deaths in women with breast cancer with IGF signalling and levels of cholesterol implicated. Metabolic syndrome and obesity are correlated with increased breast cancer risk and a worse outcome due to increased levels of insulin-like growth factors (IGFs) (Creighton *et al.*, 2008, 2012). In humans, prospective epidemiology has found a significant correlation between circulating IGF-I levels and the subsequent risk of developing several types of cancer such as colorectal, prostate and premenopausal breast cancers (Pollak, Schernhammer and Hankinson, 2004b). Several studies indicate IGF-I/IGF-II pathway in breast cancer progression (Belardi *et al.*, 2013; Mancini *et al.*, 2014).

The IGF-IR through subsequent phosphorylation of either one insulin receptor substrate-1/-2 (IRS-1/ -2) serves as a scaffold to modulate and activate other important pathway proteins as PI3K/AKT/mTOR (Mirdamadi *et al*., 2015) and Ras/Raf/MAPK (Trefely *et al.*, 2015) and this function has been well-investigated in all molecular subtypes of breast cancer (Law *et al.*, 2008). A study using global gene expression data from breast cancer patients who used neoadjuvant anastrozole treatment reported that several genes are associated with poor response to therapy such as MAPK, IGF-I and obesity (Ellis *et al.*, 2012). Activation of IGF-IR downstream signalling pathways promotes tumorigenesis by activating cell survival and proliferation and the invasiveness of cancer cells (Shukla and Gupta, 2007; Courtney, Corcoran and Engelman, 2010; Lee, Loh and Yap, 2015). Increased expression of the IGF-IR and the IR strongly associates with poor patient clinical outcomes across all molecular subtypes of breast cancer (Law *et al.*, 2008). Despite these associations, clinical results of anti-IGF-IR monoclonal antibody trials in breast cancer

have been unsuccessful (Ekyalongo and Yee, 2017) as there are currently no good biomarkers to indicate which patients will respond. Identifying potential biomarkers of response may enable a more targeted and therefore more successful approach to implement the anti-IGF-IR therapies.

Cancer cells utilise glucose as a source of energy(Garber, 2004), and the negative association of obesity and type 2 diabetes with breast tumours may be indicating an elevated energy supply that would enhance breast carcinogenesis. Cholesterol biosynthesis (intracellular) is also dysregulated in cancer cells due to increased acetyl CoA levels via glycolysis, fatty acid biosynthesis or HMG-CoA reductase activity (Kwan *et al*., 2008; Ahern *et al*., 2011), which is significantly increased or up-regulated in several cancer types including breast tumour, and has been correlated with a more aggressive breast cancers (Azrolan and Coleman, 1989).

The LDL-R is abundant on TNBC cells and is important in the growth of TNBCs in the setting of elevated circulating LDL-C (Gallagher *et al.*, 2017b). In both *in vivo* and *in vitro* models, it has been reported that the addition of LDL-cholesterol (100μg/ml) increased proliferation and migration and reduced adhesion of breast cancer cells, compared to untreated conditions. While in hypercholesterolemic mice, breast tumours were larger and more proliferative and metastasized to the lungs (dos Santos *et al.*, 2014).

There is emerging evidence to indicate that crosstalk exists between cholesterol metabolism and IGF signalling pathways. Lipid rafts are known to contain tyrosine kinase receptors, such as HER2, EGFR and the IGF-IR (Laurentiis, Donovan and Arcaro, 2007) and their signalling effects are dependent on the cholesterol abundance of the lipid-rafts (Chen and Resh, 2002). Disruption of lipid raft in TNBC breast cancer cell lines, applying

the cholesterol sequestering agent methyl-β-cyclodextrin, MβCD, reduced cell growth and induced apoptosis (Badana *et al.*, 2018). Statins are 3-hydroxy-3-methylglutharyl-coenzyme-A reductase (HMGCR) inhibitors that inhibit the rate limiting step in the production of cholesterol. Statins also impact on the pathways activated by EGF and IGF and down-regulate IGF-IR expression in prostate cancer cell lines (Sekine *et al.*, 2008).

6.2. Aims and objective

In this chapter, we aimed to delineate the involvement of the IGFs/IGF-IR in the effects of cholesterol on breast cancer cell growth and invasion.

6.3. Specific aims

Aim 1: To examine the effect of LDL and 27OHC on the levels of IGFs in MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) cells

Aim 2: To address if the effects of LDL on cell proliferation and migration in both ERα-positive and ERα-negative breast cancer epithelial cell lines were mediated through the IGF-1R.

Aim 3: To examine the effects of LDL on IGF-I-induced activation of MAPK/AKT in both ERα-positive and ERα-negative breast cancer cell lines.

6.4. Methods

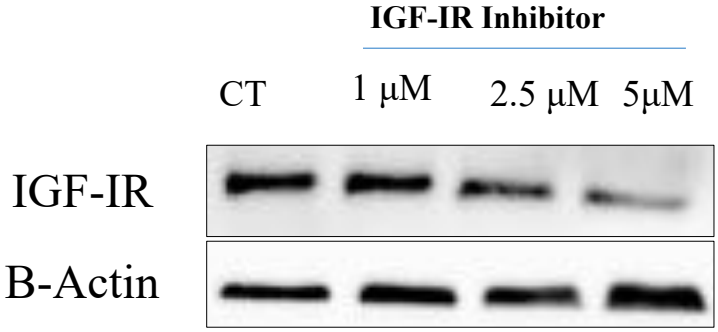
6.4.1. Cell culture:

The human breast cancer cell lines MCF7, and MDA-MB-231 were purchased from ATCC (Teddington, Middlesex, UK). All cell lines were cultured as described before in section 2.1.

6.4.2. Dosing with exogenous 27OHC, LDL and an IGF-IR antagonist:

For cell proliferation assays, cells were incubated in growth media DMEM for 24 hours, followed by 24 hours in serum-free media. The cells were then treated with different concentrations of 27OHC (0.1μM) and LDL (80 μg/ml) as optimised in chapters 3 and 4. To address the potential interactions of LDL with the IGF-I signalling pathway, we investigated the effects of LDL on cell proliferation and migration in the presence or absence of an IGF-IR inhibitor (AG1024). Different strategies have been developed to target this system, such as monoclonal antibodies and small molecule inhibitors, targeting the tyrosine kinase domain of the receptor(Christopoulos, Corthay and Koutsilieris, 2018b). Firstly, I investigated the optimum concentration of a tyrosine kinase inhibitor (AG1024), by using a range of concentrations (0-5μM) for both MCF-7 and MDA-MB-231; an effective reduction of the IGF-IR was shown by Western blotting (Fig 6.1). Figure 6.1 shows that based on loss of the IGF-IR, 5μM was the optimum dose for MCF-7 cells, with 2.5μM being the optimum dose for MDA-MB-231 cells (A and B respectively).

A. MCF-7



B. MDA-MB-231

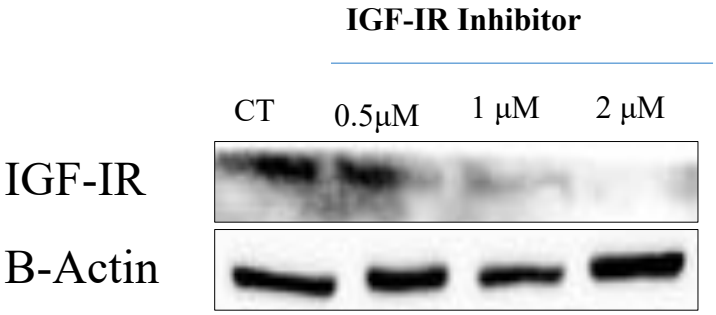


Figure 6. 1: The optimum concentration of IGF-IR inhibitor in MCF-7 and MDA-MB-231 cell lines:

A) for MCF-7 and B) MDA-MB-231 dosed with an IGF-IR inhibitor (0-5 μM) for 24hrs. The protein abundance of the IGF-IR was measured, and β-actin was used as loading control in MCF-7 and MDA-MB-231 Data representative of (n=1).

6.4.3. Radioimmunoassay (RIA)

Conditioned media levels of IGF-I were assessed using a radioimmunoassay as described in section (2.12).

6.4.4. Cell proliferation

This was determined using a CV proliferation assay (Feoktistova, Geserick and Leverkus, 2016a), as described in section 2.4.

6.4.5. Trans-well migration assay

Cells were prepared to migrate into the chemoattractant serum in the lower compartment as described in section 2.9, for 24 h (MCF-7) or 6 h (MDA-MB-231) with or without the addition of an IGF-IR antagonist (2.5-5μM) or LDL (100μg/ml).

6.4.6. Western blot

Western blot analysis was performed as described previously in section (2.10). In brief, protein cell lysates (30μg), were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane and immunoblotted with the following antibodies: anti-IGF-IRβ (1:1000), MAPK and phospho-MAPK (1:1000; Thermo fisher), Phospho-IGF-I Receptor β (1:1000; Thermo fisher), AKT (1:1000; Cole-Parmer Scientific experts), phospho-AKT(1:1000; Star Lab) GAPDH (1:5,000; Millipore) and β-actin (1:10,000; Sigma-Aldrich). After that the membranes were incubated with particular secondary antibodies (anti Mouse or Rabbit) conjugated to peroxidase (Sigma),

next proteins were detected by Clarity ECL substrate (BioRad) by using BioRad Chemidoc XRS + system and quantified using Image J 1.46r software (BioRad).

6.4.7. Targeted CYP27A1 gene knockdown:

For experiments investigating the effects of LDL(80 μg/ml) on the production of IGF-I in the presence or absence of CYP27A1 in MCF-7 and MDA-MB-231 cells, siRNA was used for silencing the CYP27A1 as described in section 3.4.

6.4.8. Statistical analysis

The whole experiments were repeated in triplicate, and also each experiment was repeated three times. Using GraphPad Prism 8.0.1 software for windows (La Jolla, CA, USA), to analyse the data, one-way ANOVA following the least significant difference (LSD) post-hoc test.

6.5. Results

6.5.1. The effect of LDL and 27OHC on the levels of IGF-I in MCF-7 and MDA-MB-231 cells

As 27OHC and LDL induced migration and invasion in breast cancer cell lines, we next examined the effect of 27OHC on the IGF-I signalling pathway in both ERα-positive and ERα-negative breast cancer cell lines. Treatment with 27OHC (0.1μM) and LDL(80 μg/ml) for 48hrs, increased the abundance of the IGF-IR in MCF-7 cells ($p<0.001$ and $p<0.01$ respectively) (Fig. 6.2A and B) and in and MDA-MB-231 ($p<0.01$ and $p<0.001$ respectively) (Fig. 6.2C and D) in comparison to the controls. With the radioimmunoassay, the addition of 27OHC (0.1μM) and LDL(80 μg/ml) increased the production of IGF-I in MCF-7 ($p<0.05$ and $p<0.001$) respectively and increased the production of IGF-I in MDA-MB-231 cells ($p<0.01$ and $p<0.001$) respectively (Fig 6.3). These results suggest that there is a link between IGF and cholesterol metabolism.

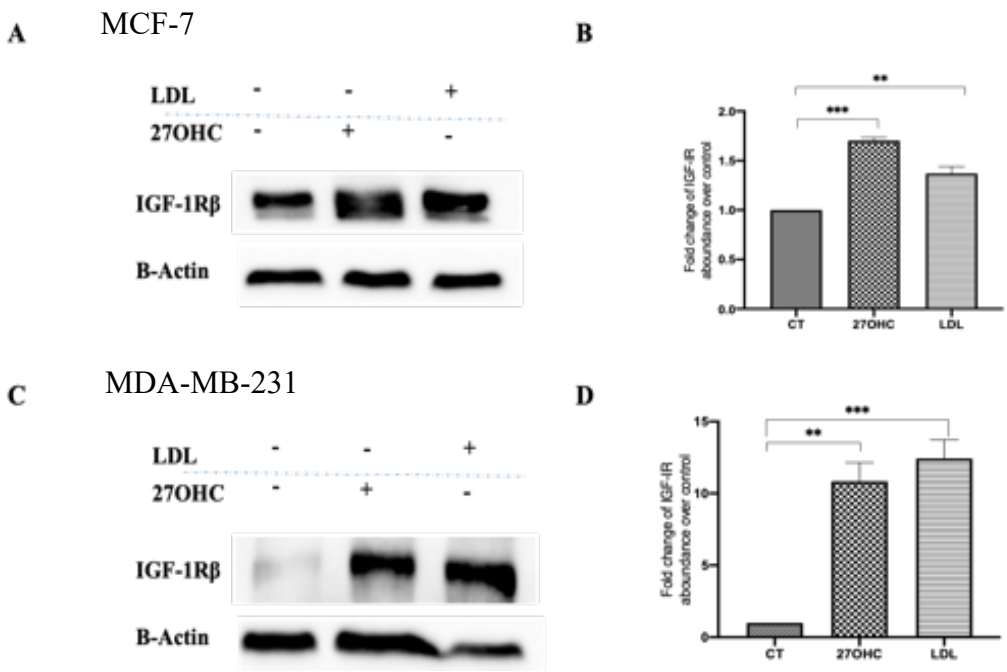


Figure 6. 2: The protein level of IGF-IR was determined using western blotting:

*western blotting for **A)** MCF-7 and **C)** for MDA-MB-231 after being dosed with 27OHC (0.1µM) and LDL(80 µg/ml) for 48hrs. Densitometry analysis of fold changes of IGF-IR against loading control β-actin in B) MCF-7 and D) MDA-MB-231. Data representative of mean ±SEM (n=3). P-values were determined using GraphPad Prism one-way Statistical analysis: ANOVA test plus least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

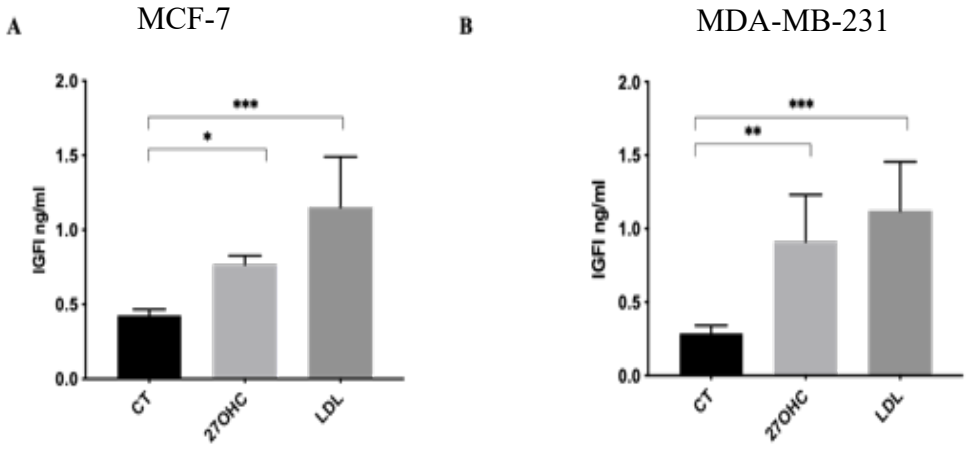


Figure 6. 3: Radioimmunoassay to measure IGF-I concentrations:

*in **A)** MCF-7 and **B)** MDA-MB-231cells after being dosed with 27OHC (0.1µM) and LDL (80 µg/ml) for 48 hours. Data are representative of mean ±SEM (n=3). P-values were determined using GraphPad Prism one-way Statistical analysis: ANOVA test plus least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001*

6.5.2. The effects of LDL on cell proliferation and migration in the presence or absence of an insulin-like growth factor receptor antagonist

To address the potential interactions of LDL with the IGF-I pathway, the effects of LDL on cell proliferation and migration in the presence or absence of the IGF-IR were investigated. We used a tyrosine kinase inhibitor, AG1024, to inhibit the IGF-IR for both MCF-7 and MDA-MB-231. Cells were treated with LDL (80 µg/ml) for 48hrs, then changes in growth and migration were assessed. Figure 6.4A shows that LDL treatment increased MCF-7 proliferation ($p < 0.001$) in comparison to the control, whereas in the presence of the IGF-IR antagonist, LDL was unable to increase cell growth. LDL treatment significantly increased MCF-7 cell migration ($p < 0.001$), and this effect of LDL was also lost in the presence of the IGF-IR antagonist (Fig. 6.4B).

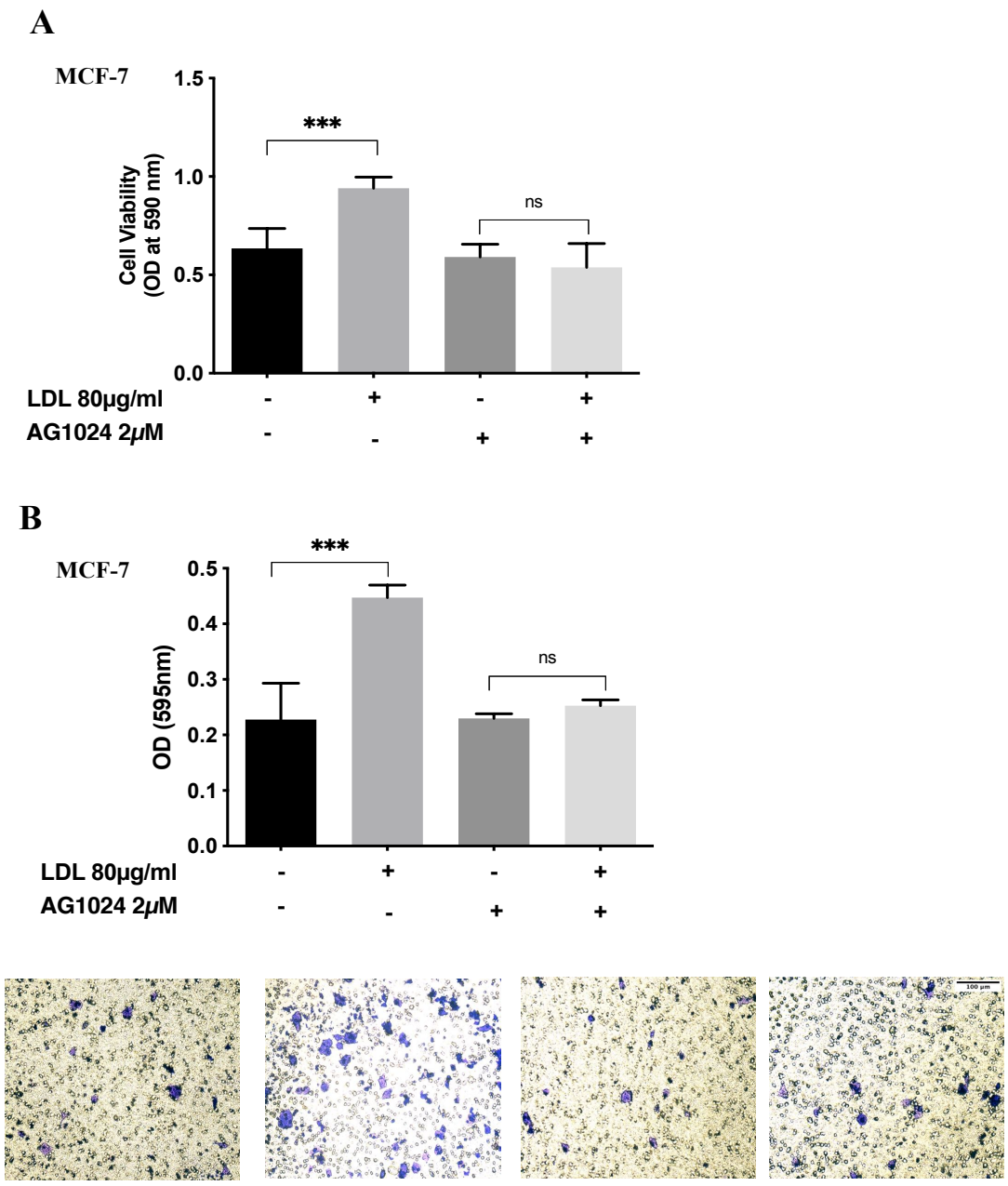


Figure 6. 4: The effects of LDL on cell proliferation and migration in the presence or absence of an insulin-like growth factor receptor antagonist:

*Cell proliferation for A) MCF-7 treated with (LDL 80µg/ml) in the presence or absence of a tyrosine kinase inhibitor (AG1024;5µM) for 48 hours, by using crystal violet proliferation assay. Cell migration for B) MCF-7 cells after being dosed with LDL (80µg/ml) with or without AG1024 (5µM). The migrated cells were stained with crystal violet and images were taken (×20 magnification). Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test plus least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001). Scale bar represents 100µm.*

The same results were found with MDA-MB-231: Figure 6.5A shows that LDL (80μg/ml) treatment increased cell proliferation ($p < 0.001$) in comparison to the control, whereas with the IGF-IR (2μM) antagonist, LDL was no longer able to increase cell growth. The ability of the LDL to increase MDA-MB-231 migration was also significantly inhibited in the presence of the IGF-IR antagonist (Fig. 6.5B). Inhibition of the insulin-like growth factor receptor using a tyrosine kinase inhibitor, AG1024, blocked the effects of cholesterol on cell growth and invasion of MCF-7 and MDA-MB-231 cells.

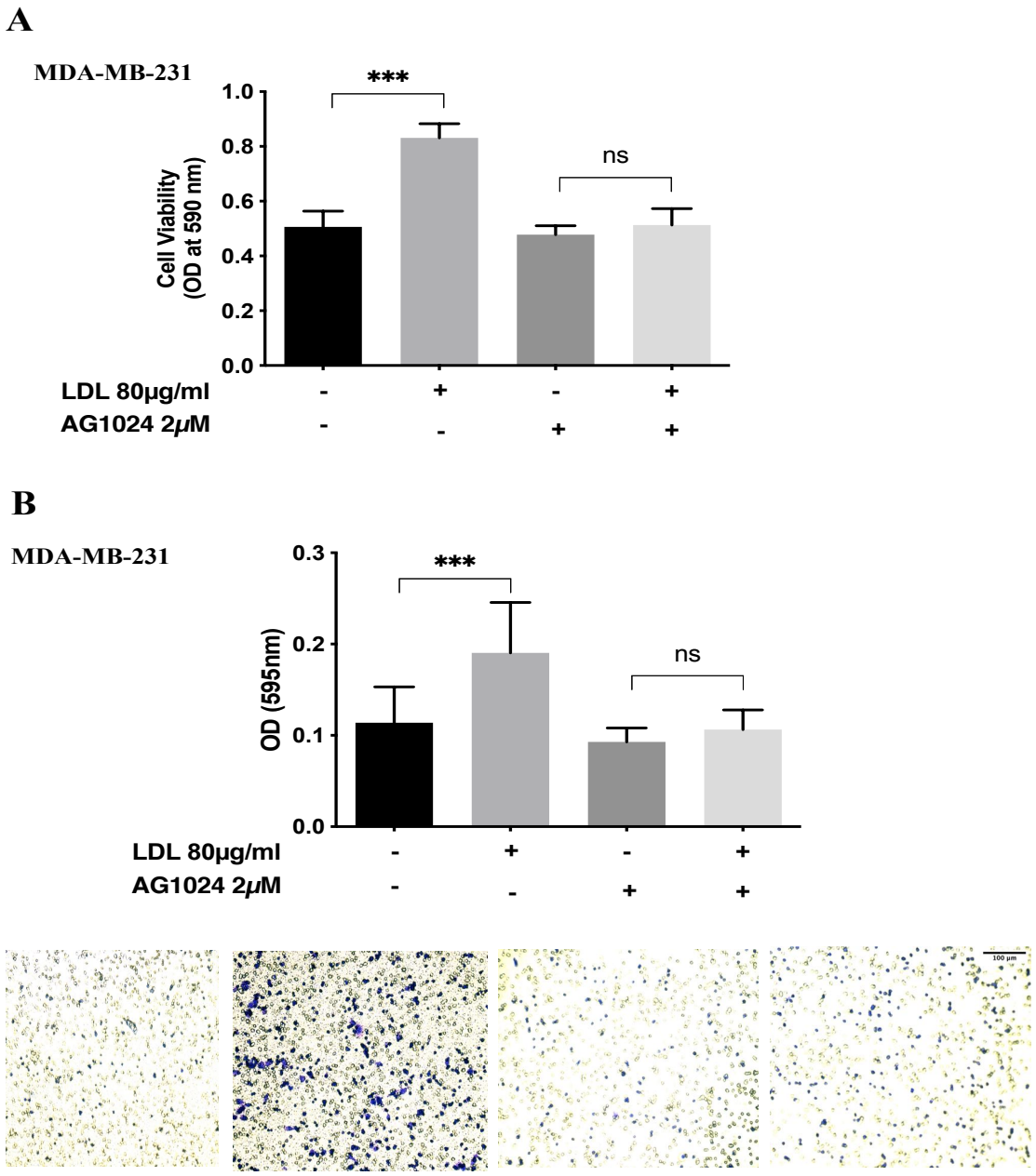


Figure 6. 5: The effects of LDL on cell proliferation and migration in the presence or absence of an insulin-like growth factor receptor antagonist:

*The cell proliferation for A) MDA-MB-231 after being treated with (LDL 80µg/ml) with or without a tyrosine kinase inhibitor (AG1024 2µM) for 48 hours, by using CV proliferation assay. Trans-well migration assay was used to perform the cell migration for B) MDA-MB-231 after being dosed with (LDL 80µg/ml) in the presence or absence of AG1024 (2µM): the migrated cells were stained with CV and images were taken (×20 magnification). Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism; ANOVA test plus least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001). Scale bar represents 100µm.*

6.5.3 . The effect of LDL on the MAPK/AKT transduction pathways in the presence or absence of an insulin-like growth factor receptor antagonist

In MCF-7 cells, the LDL-induced increase in MAPK and AKT activation *($p < 0.01$)* was blocked when the insulin-like growth factor receptor was disrupted using a tyrosine kinase inhibitor (Fig. 6.6A-C). The western blots show effective downregulation of the IGF-IR and inhibition of phospho-IGF-IR (*$p < 0.001$*) in MCF7 cells (Fig. 5.6B and C). Similarly, with MDA-MB-231 cells, LDL-induced an increase in the phosphorylation of MAPK and AKT and these effects were inhibited when the insulin-like growth factor receptor was blocked using a tyrosine kinase inhibitor (Fig. 6.7). This indicates that the inability of LDL to enhance activation of MAPK/AKT pathway upon the disruption of IGF-IR suggesting that the IGF-IR is required for MAPK and AKT activation induced by LDL.

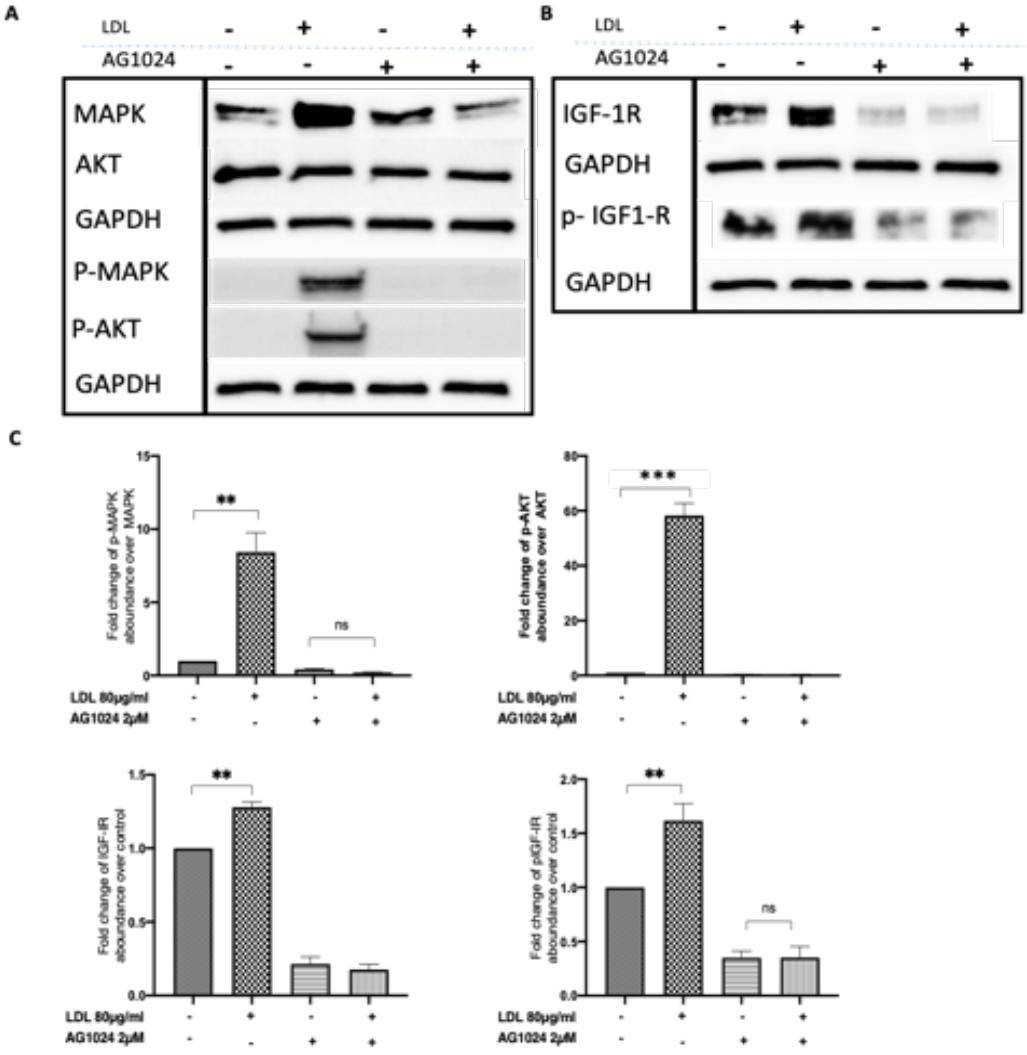


Figure 6. 6: Levels of the IGF-IR, p-IGF-IR, MAPK, p-MAPK, AKT, p-AKT were determined using western blotting in the presence or absence of LDL with or without an insulin-like growth factor receptor antagonist:

*A&B) MCF-7 cells, after being treated with (LDL 80µg/ml) and a tyrosine kinase inhibitor (AG1024 2µM) for 48 hours, were assessed the levels of IGF-IR, p-IGF-IR, MAPK, p-MAPK, AKT, p-AKT and GAPDH was used as a loading control for IGF-IR and p-IGF-IR, but AKT and MAPK for p-AKT and p-MAPK respectively. C) Densitometry analysis of fold changes of IGF-IR against loading control β-actin, MAPK against p-MAPK and AKT against p-AKT. Data representative of mean ±SEM (n=3). P-values were determined by using GraphPad Prism one-way Statistical analysis: ANOVA test plus least significant difference (LSD) post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).*

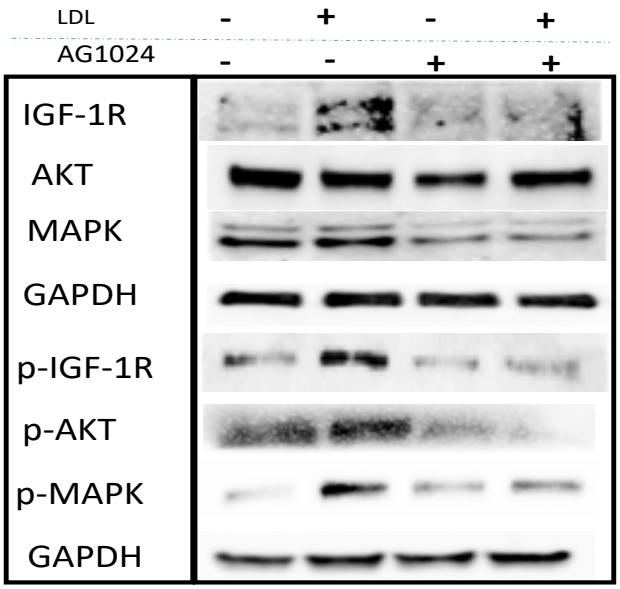


Figure 6. 7: The levels of the IGF-IR, p-IGF-IR, MAPK, p-MAPK, AKT, p-AKT were determined using western blotting of LDL in the presence or absence of an insulin-like growth factor receptor antagonist:
in A&B) MDA-MB-231 cells, C) Densitometry analysis of fold changes of IGF-IR against loading control β-actin, p-MAPK against MAPK and p-AKT against AKT. Data representative of mean ±SEM (n=2).

In chapter 3, it was shown that LDL induced the proliferation, invasion and migration of both MCF-7 (ERα-positive) and MDA-MB-231 (ERα-negative) cells and this was inhibited when the enzyme that converts cholesterol into 27OHC (CYP27A1) was silenced using siRNA.

Furthermore, it was determined that the effects of LDL on the production of IGF-I in the presence or absence of CYP27A1 were inhibited in both ERα-positive (Fig. 6.8A) and negative (Fig. 6.8B) breast cancer cell lines. It also reduced the abundance of the IGF-IR in MCF-7 cells (Fig. 6.8C). These data suggest that CYP27A1 is a positive regulator of IGF-I receptors.

Interestingly, using the publicly available METABRIC gene expression database, we also demonstrated that tumours with high levels of CYP27A1 (and thus expected elevated 27OHC) also had increased expression of IGF-I (*p <0.001*) (Fig. 6.8C). Conversely low tumour expression of CYP27A11 was associated with lower expression of IGF-I (Fig. 6.8C).

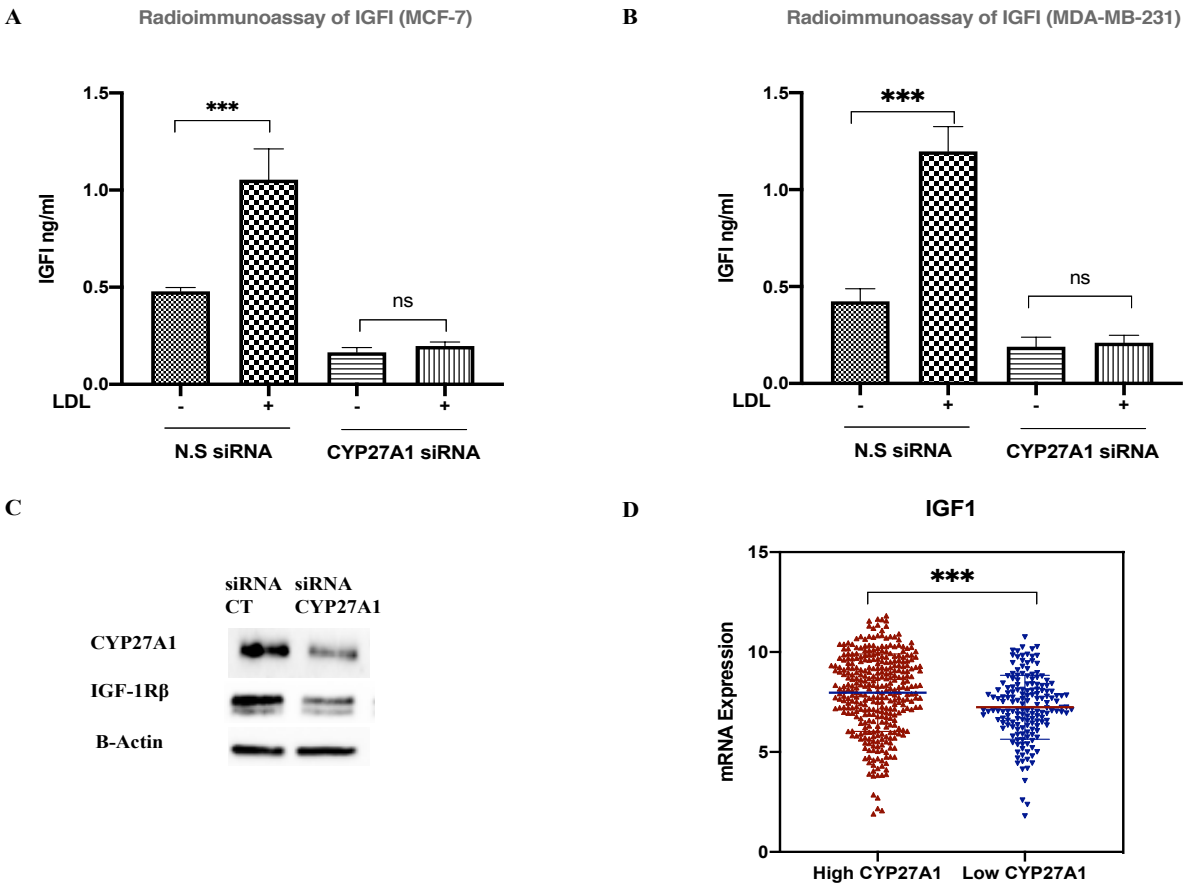


Figure 6. 8: Levels of IGF91 in the presence or absence of CYP27A1:
*in A) MCF-7 and B) MDA-MB-231 after being dosed with LDL (80µg/ml) in the presence or absence of CYP27A1 for 48 hours. C) The protein expression of the IGF-IR and CYP27A1 were assessed using western blot analysis. GAPDH was used as a loading control. D) Scatterplot analysis of TCGA data of 504 invasive breast cancer carcinomas for mRNA expression of IGF-I represented as median expression of CYP27A1. Results are presented as mean +/− SEM (n=3) (A-C), and D) where the horizontal line presents the mean. P-values were determined by using GraphPad Prism: one-way Statistical analysis: one-way ANOVA test followed by Tukey's post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001) (A-C), and D) Mann–Whitney test.*

6.6. Discussion

Growth factor signalling is often deregulated in cancer cells. One of the most common activators of PI3K and its downstream target Akt is IGF-I via its receptor IGF-IR, which can modulate cell survival (Chimento *et al.*, 2019b). In overweight women, it has been shown that IGF signalling and levels of cholesterol, specifically LDL-C are up-regulated. Recently it has been found that LDL-R abundance is important in the growth of TNBCs in the setting of increased circulating LDL-C and is thought to be a major contributing factor to the increased mortality and recurrence in overweight women with TNBC (Gallagher *et al.*, 2017b). Recent meta-analyses reported that breast cancer patients who use statins, show improved recurrent-free, overall and cancer- specific survival (Manthravadi, Shrestha and Madhusudhana, 2016; Li *et al.*, 2018).

We found that the addition of LDL increased the production of IGF-I by MCF-7 and MDA-MB-231 cells. It also increased the abundance of the IGF-IR in MCF-7 and MDA-MB-231 cells, and this phenotype has been confirmed recently in prostate cancer, PC-3 cells. These data indicated that simvastatin treatment deregulated IGF-IR expression (Sekine *et al.*, 2008) and impeded both basal IGF-I and IGF-I-induced ERK and Akt activation (Sekine *et al.*, 2018). Breast cancer (MCF-7, MDA-MB231) and prostate cancer (PC-3, LNCaP) cells are more sensitive to cholesterol sequestering than normal-like prostate and breast cell lines (PZ-HPV7 and MCF-10A, respectively) (Li *et al.*, 2006). In fact, raft modulating agents are more efficient in cells with high content of lipid rafts, such as, breast cancer cell lines MCF-7 and MDA-MB231, than normal-like breast cell lines (Sekine *et al.*, 2018).

The data also clearly indicate that LDL increased the proliferation of MCF-7 and MDA-MB-231 cells and increased their cell invasion. Inhibition of the insulin-like growth factor

receptor using a tyrosine kinase inhibitor, AG1024, blocked the effects of cholesterol on cell growth and invasion of MCF-7 and MDA-MB-231 cells. The inhibition of migration and growth that we found was consistent with results in the other reports in different cell lines (Li *et al.*, 2014).

Statins can influence the signalling pathways activated by IGF and EGF. Simvastatin has been proposed to inhibit the IGF-IR in bile duct cancer cells (Laurentiis, Donovan and Arcaro, 2007), suppress esophageal cancer cell growth and can lead to the development of radioresistance by reversing the process of epithelial-to-mesenchymal (EMT) transition through the PTEN-PI3K/AKT signalling pathway and this phenotypic effect was blocked by the addition of IGF-I (Li *et al.*, 2014).

Several data suggest that statins reduce tumour growth and migration (Livingstone *et al.*, 2014; Cardwell *et al.*, 2015; Borgquist *et al.*, 2019), deregulate IGF-1R expression (Sekine *et al.*, 2008) and inhibit both basal IGF-I and IGF-I-induced ERK and Akt activation (Sekine *et al.*, 2018). Conversely, it has been shown that alteration of circulating cholesterol using high cholesterol diets in mice, promoted prostate tumor proliferation and survival, as a result of activated Akt signalling pathway through cholesterol-rich lipid rafts (Zhuang *et al.*, 2005). Although MAPK pathway has been associated with growth and survival responses, we indicated that after 48hrs of treatment with LDL, both AKT and MAPK were activated, and these effects of cholesterol were blocked with an IGF-IR inhibitor. The inhibition of insulin-like growth factor-1 receptor attenuated cholesterol-induced AKT and MAPK activation. This suggests that AKT and MAPK are associated with LDL actions in breast cancer cell lines. The same effect has been investigated but in different cancer type such as prostate cancer cell lines (Sekine *et al.*, 2018). Activated

PI3K/Akt controls cell survival, growth and tumorogenesis, and abnormal activation of this pathway participates in the invasiveness and development of cancer cells (Shukla and Gupta, 2007; Courtney, Corcoran and Engelman, 2010).

There have been different explanations for the mechanism by which cholesterol associates with the IGF-I signalling pathway (Araya, Tang and Wikvall, 2003; Sekine *et al.*, 2008). *In vitro* experiments in human cells, hepatocytes (HepG2) cells, reported a role for growth hormone, IGF-I, and glucocorticoids in the regulation of the enzyme CYP27A1 activity (Araya, Tang and Wikvall, 2003). Considering this it is interesting that LDL increased the production and secretion of IGF-I and this was inhibited when the enzyme CYP27A1 was silenced in both ERα-positive and -negative breast cancer cell lines. It also reduced the abundance of the IGF-IR in MCF-7 cells. These data were consistent with the results from the publicly available METABRIC gene expression database, which together suggested that CYP27A1 is a positive regulator of IGF-I receptors. These phenotypic effects that we observed have been confirmed recently by others with different methods of targeting cholesterol metabolism (Laurentiis, Donovan and Arcaro, 2007; Lee *et al*., 2016). There are some limitations; we evaluated the association of MAPK and AKT pathway with the action of LDL in breast cancer cell lines, but we did not investigate if AKT or MAPK were involved in the effects of LDL and 27OHC. The data show that blocking the IGF-1R with a tyrosine kinase inhibitor, inhibited the effects of LDL on activating AKT and MAPK, and its ability to increase levels of the IGF-1R and IGF-I. Blocking CY27A1, had similar effects, suggesting that LDL via 27OHC is a positive regulator of IGF signalling. Considering the upregulation of other membrane proteins in the previous chapter, such as CAV1 and the beta 1 integrin, it would be interesting to investigate if an association existed

between these molecules at the cell membrane and if this played a part in regulating the downstream signalling that we observed. Since IGF-1R activation needs ligand binding that lead to elevate IGF-1 levels, decreasing IGF1 level may be a reasonable approach for future to test the potential hypothesis that LDL promotes IGF1 expression which in turn activates the IGF1R pathway.

6.7. Conclusion

Our data suggest that the actions of LDL on the growth, invasion and activation of the AKT/MAPK pathway of breast cancer cells are mediated by the IGF-IR. These results advise that the combination of cholesterol lowering drugs and agents targeting the IGF-IR may be an effective strategy for the treatment of breast cancer.

Chapter 7.

General discussion

7.1. General discussion

Epidemiological studies indicate that cholesterol metabolites have a role in enhancing tumour growth and invasion and increase the risk of breast cancer in postmenopausal women (DuSell *et al.*, 2008; Wu *et al.*, 2013; Nguyen *et al.*, 2015; He and Nelson, 2017). This is probably due to elevated oxysterol production which associates with hypercholesteremia. Increased concentrations of cholesterol metabolites have also been associated with breast cancer (Nelson *et al.*, 2013a; Wu *et al.*, 2013; Gallagher *et al.*, 2017a). Cholesterol-lowering drugs (such as statins) have a protective effect against breast cancer deaths and recurrence (Beckwitt *et al.*, 2018), suggesting that lowering cholesterol and 27OHC may have therapeutic benefit against breast cancer development. Nevertheless, the definite mechanism as to how cholesterol affects breast cancer pathogenesis, particularly with regard to the ERβ, is still not clear (Lipovka and Konhilas, 2016).

We evaluated the significance of high cholesterol-LDL on the growth and migration of breast cancer cell lines and found that LDL drives the proliferation of ERα-positive and ERα-negative cells, and this is consistent with a previous study examining the importance of the LDL-R in the growth of TNBCs (Gallagher *et al.*, 2017a). The inhibition of CYP27A1 blocked the action of LDL, suggesting that the exogenous of 27OHC can contribute to the risk of breast cancer by mediating the effect of cholesterol LDL.

The phenotype that we observed has been confirmed by others (Nelson *et al.*, 2013a; Wu *et al.*, 2013) in addition to clinical evidence supporting a role of the 27OHC/CYP27A1 metabolic signalling pathway in breast cancer (Nelson *et al.*, 2013a; Baek *et al.*, 2017; Le Cornet *et al.*, 2020). Moreover, a recent study has demonstrated the correlation between CYP27A1 expression, high grade tumours, and an increased risk for aggressive disease

which suggests that CYP27A1 can be a biomarker of late lethal disease in postmenopausal women who have ER-positive breast tumours (Kimbung *et al.*, 2020). Additionally, a study in bladder cancer cells showed that blocking CYP27A1 can reduce the cellular cholesterol levels in androgen receptor-positive cell lines (Liang *et al.*, 2019) and the same result was found in prostate cancer cell lines (Alfaqih *et al.*, 2017).

LDL induced proliferation in ERα-negative cells, MDA-MB-231, but despite siRNA to CYP27A1 blocking the effects of LDL, exogenous addition of 27OHC did not induce proliferation. There are other metabolites of cholesterol that can also mediate effects of cholesterol. This may mean that the effects of LDL in MDA-MB-231 cells may be mediated through other metabolites. 25-hydroxycholesterol has been shown to promote bladder cancer cell proliferation and the EMT markers in human bladder cancer cells (Wang, He and Fang, 2020). Therefore, it will be helpful for future work to investigate how CYP27A1 impacts breast cancer progression as it may be a useful strategy to treat or prevent breast cancer. These data indicate that cholesterol lowering treatments such as statins could also be a good strategy to control the risk of breast cancer, since it has been reported that statins can decrease 27OHC concentrations in breast cancer tumours (Kimbung *et al.*, 2017b).

We found that 27OHC promoted cell growth in ERα positive, but not in ERα negative cell lines, and this effect was mediated via the ERα, as has been observed previously (DuSell *et al.*, 2008; He and Nelson, 2017; Starkey *et al.*, 2018). Interestingly, we found that 27OHC was able to promote breast cancer cell migration and invasion in both cell lines. We elucidated that the proliferative effects of 27OHC involved the ERα and that perhaps

targeting ERα with lowering cholesterol treatment in ERα-positive breast cancer cells could be a targeted approach for decreasing breast cancer growth.

However, silencing the ERα in the ERα-positive cells had no effect on the ability of 27OHC to promote migration and invasion. In an attempt to understand what was mediating the effects of 27OHC, we silenced the ERα in the ERα-positive cells and observed an increase in the abundance of the ERβ. It is consistent with previous data whereby the role that 27OHC plays depends on the relative ratio of expression of ERα to ERβ (Leygue and Murphy, 2013), and this suggested that ERβ can mediate the metastatic actions of 27OHC in the absence of ERα. High expression of ERβ in TNBCs and its structural similarity to ERα have suggested that it is a logical therapeutic target (Abe *et al.*, 1998; Novelli *et al.*, 2008a). There have been conflicting findings about the role of ERβ in breast cancer (Jensen *et al.*, 2001; Hamilton *et al.*, 2015; Bialesova *et al.*, 2017; Austin *et al.*, 2018), due to differences between the studies; this could relate to the lack of standardized detection methods and insufficiently validated antibodies (Carder *et al.*, 2005). Moreover, ERβ has five different isoforms, which could also complicate the investigation of the physiological role of ERβ and its participation in the carcinogenesis of breast cancer (Girgert, Emons and Gründker, 2019). It has been shown that ERβ1, ERβ2 and ERβ5 have been expressed in breast cancer tissue (Yan *et al.*, 2020). To address these differences we used a certified TNBC cell line (negative for ERα. PR, and HER2 overexpression) and validated ERβ antibodies (Andersson *et al.*, 2017). Moreover, we have successfully used three different strategies to silence ERβ in MDA-MB-231 TNBC cells.

In future studies, it will be important to determine the ratio of ERβ1, ERβ2 and ERβ5 in a large sample of TNBC and to associate the cholesterol/oxysterol level with treatment outcome (Murphy and Leygue, 2012).

We found that 27OHC-induced cell migration and invasion and increased the abundance of an EMT marker fibronectin, through ERβ, suggesting that 27OHC can act via both the ERα and ERβ in our models, dependent on the context. This is supported by the observation that 27OHC increases cell proliferation in prostate cancer cell lines via ERβ activation (Raza *et al.*, 2017). This is also in agreement with a previous study indicating that 27OHC can promote breast cancer migration and invasion and EMT markers (Jiao *et al.*, 2020) and that ERβ suppression decreased the expression of vimentin and fibronectin, and decreased MDA-MB-231 breast cancer cell migration and invasion (Piperigkou *et al.*, 2016). In contrast, recent studies indicate that 27OHC acts as a negative modulator of ERβ in Ishikawa uterine cancer cells, and they explained that this effect was because of the concentration of etradiol (E2) was higher than in previous reports (Starkey *et al.*, 2018). They suggested that 27OHC may function as a competitive ligand in smooth muscle and vascular endothelial cells (Umetani *et al.*, 2007). Since 27OHC shows both agonistic and antagonistic actions on ERα functions depending on target organs (Umetani, 2016), 27OHC may also function as both an antagonist and agonist for ERβ dependent on specific tissues/cells or the presence or absence of the ERα (Skliris *et al.*, 2006b). These novel results and the previous studies suggest that endocrine treatment in combination with lowering cholesterol drugs may be beneficial for improving ER-positive breast cancer outcomes. As a previous study showed, a combination of cholesterol lowering drug and

adjuvant endocrine therapy may improve the outcome of patients with ER-positive breast cancer (Borgquist, Giobbie-Hurder, Thomas P. Ahern, *et al.*, 2017).

In TNBC, ERβ1 expression associates with Ki-67, a cell growth biomarker, indicating that ERβ1 can have a role in driving growth (Yan *et al.*, 2013), and this is consistent with the results from the TCGA database, in which we found that tumours with high mRNA levels of ERβ also had increased expression of Ki-67.

Functional crosstalk exists between ERs and growth factor receptors, such as EGFR and IGF-IR. Growth factor pathways can activate estrogen, and estogen can activate IGF signalling (Yee and Lee, 2000). Treatment of ERα-positive breast cancer cells with tamoxifen can lead to resistance, and IGF-IR and/or EGFR are critical for breast cancer resistance to endocrine therapy (Ignatov *et al.*, 2010). The role of ERβ in breast cancer growth was shown in ERα-negative tumours, but not ERα-positive tumour (Yan *et al.*, 2013). Additionally, ERβ associates with cancer growth and progression in lung cancer(Hershberger *et al.*, 2009) and prostate cancer (Raza *et al.*, 2017).

With ERβ inhibition, we observed a suppression of IGF-I and EGF receptors and an inhibition of basal cell proliferation. The phenotype that we observed has been confirmed recently by others in TNBC, but they observed an interaction between ,ERβ and insulin-like growth factor-II (IGF-II) in TNBC(Hamilton *et al*., 2015; Piperigkou *et al*., 2016). Conversely, we used a different approach to confirm this finding and found that up-regulating ERβ, using an agonist, increased levels of the IGF-I and EGF receptors in MDA-MB-231 cells. These data indicate that ERβ is a positive regulator of the IGF-I and EGF receptors. A link between ERβ with IGF and EGF signalling pathways has also been reported previously where silencing ERβ in TNBC cell lines reduced cell proliferation,

migration and invasion that was associated with down-regulation of the IGF-I and EGF receptors (Richardson *et al.*, 2011; Hamilton *et al.*, 2015). Some studies have indicated that ERβ found to be a biomarker linked to a more aggressive breast cancer (Novelli *et al.*, 2008a).This is consistent with the results from the TCGA database, in which we found an association between the levels of ERβ with IGF-IR and EGFR mRNA levels and that may suggest that ERβ is a positive regulator of IGF-IR, EGFR and Ki-67. We elucidated that the phenotypic effects of LDL and 27OHC involved the IGF-IR and EGFR and that perhaps targeting ERβ in TNBC could be a different approach for down-regulating IGF signalling.

The wealth of pre-clinical studies indicate a role for EGF and IGF signalling pathways in TNBC tumours, and these pathways are correlated with poor response to therapy, reduced overall survival and higher rates of recurrence (Cox *et al.*, 2015; Christopoulos, Corthay and Koutsilieris, 2018a). Several strategies were developed to target the IGF and EGF signalling pathway and these strategies were applied as monotherapies and had limited success(Christopoulos, Corthay and Koutsilieris, 2018b). This indicated the need to identify a good biomarker to be able to select those patients most likely to respond. Based upon our results, we suggest that potentially the ERβ may be investigated as biomarker of response to anti-IGF-IR and EGFR therapies, such as inhibitors of the tyrosine kinase domains of these receptors (TKIs).

In the presence of 27OHC or LDL, doxorubicin was less effective in inducing cell death, suggesting they can act as survival factors in MDA-MB-231, TNBC. This result was consistent with previous work where doxorubicin significantly downregulated HMG-CR, and this led to a decrease in the level of the EGFR, and thus enhanced doxorubicin-induced

cell death. In addition, knockdown of HMG-CR in the presence of DOX enhanced PARP cleavage, which is an indicator of caspase-3 activation and consequent apoptosis (Yun *et al.*, 2019). We determined that the phenotypic effects of LDL involved the IGF and EGF signalling, and as previous studies have demonstrated, this pathway is associated with poor response to therapy. We suggested that the phenotypic effects of LDL decreasing doxorubicin cytotoxicity, may involve the IGF and EGF signalling pathways. Therefore, perhaps inhibiting cholesterol metabolism in TNBC may be an effective approach in addition to chemotherapy treatment to lower the dose and reduce the side effects of chemotherapy treatment. The same result was reported for MCF-7 breast cancer cells, and it indicated that simvastatin reduced cell growth and increased the rate of cell death in doxorubicin treated cells, and it has been suggested that simvastatin may promote doxorubicin cytotoxicity in ERα-positive breast cancer cells (Buranrat, Suwannaloet and Naowaboot, 2017).

Depleting or sequestering cholesterol in cells from the membrane leads to a shift in raft and hormonal receptors and caveolar proteins to other parts of the plasma membrane (Garnett and Greenhough, 2013). We found that the addition of 27OHC increased the abundance of CAV1, the β1-integrin and the IGF-IR. In our laboratory, Zielinska *et al.* have indicated that CAV1 is a marker of an invasive, hormone-resistant phenotype (Zielinska *et al.*, 2018) and that CAV1 associates with the β1 integrin in TNBC cells (Burrows *et al.*, 2006). Moreover, it has been indicated that CAV1 regulates IGF-I and EGF-R signalling. (Laurentiis, Donovan and Arcaro, 2007). Since we determined that the effects of LDL involved modulation of IGF and EGF, it will be helpful in future study to determine if

CAV1, EGFR and IGF-IR play an important role in the phenotype induced by LDL and 27OHC.

IGF signalling and levels of cholesterol are frequently up-regulated in overweight women and this is thought to be important contributing factors to the increased mortality and recurrence in overweight women with TNBC breast cancer(Gallagher *et al.*, 2017b). The insulin family of growth factors mediates effects of metabolic disturbances (Belardi *et al.*, 2013). IGF expression is strongly increased in invasive breast cancer through downstream activation of the mTOR pathway leading to TNBC cell migration (Mancini *et al.*, 2014). Our findings show that the addition of LDL increased the production of IGF-I and the abundance of the IGF-IR in MCF-7 and MDA-MB-231 cells. The same phenotype has been confirmed recently in breast cancer patients, these data indicated that the increase of serum IGF-I is correlated with high levels of metabolic syndrome markers, including LDL-cholesterol, triglyceride and high BMI and this increased the risk of breast cancer progression (Khaddour *et al.*, 2020). Moreover, simvastatin treatment deregulated IGF-IR expression through inhibition of the AKT pathway (Sekine *et al.*, 2008, 2018) and inhibited both basal IGF-I and IGF-IR expression (Sekine *et al.*, 2018). We found that inhibition of the IGF-IR blocked the effects of cholesterol on cell growth and invasion of MCF-7 and MDA-MB-231 cells consistent with other reports in different cell lines (Li *et al.*, 2014). The same results were found in bile duct cancer cells, with a report that the statin, simvastatin was able to influence the signalling pathways activated by IGF and EGFR. (Laurentiis, Donovan and Arcaro, 2007). Moreover, in a prostate cancer cell line, PC-3s, simvastatin inhibited cell proliferation and migration through inhibiting activation of the

IGF-I/AKT pathway (Sekine *et al.*, 2018). This suggests that cholesterol is associated with the IGF-I pathway to promote cancer proliferation, migration, and invasion.

My study indicated that inhibition of the IGF-IR attenuated cholesterol-induced AKT and MAPK activation. This suggests that AKT and MAPK are associated with LDL actions in breast cancer cell lines. The same effect has been reported previously (Sekine *et al.*, 2018). Activated PI3K/AKT controls cell survival, proliferation and tumorogenesis, and the abnormal activation of the PI3K/AKT pathway due to the development and invasiveness of cancer cells (Shukla and Gupta, 2007; Courtney, Corcoran and Engelman, 2010). These data are consistent with our results from the TCGA gene expression database, and this implies that the CYP27A1 may function as a positive regulator of IGF-I receptors. The effects that we observed have been confirmed recently by others with different methods of targeting cholesterol metabolism (Laurentiis, Donovan and Arcaro, 2007; Lee *et al*., 2016). It will be a helpful in future work, performing inhibitor studies, to investigate if AKT or MAPK pathways are involved and not just associated with their action in the effects of LDL and 27OHC. There are some limitations: we evaluated the effects of 27OHC and LDL on changes of gene expressions only at a single time point instead of performing a time-course experiment. Although, we selected breast cancer cell lines, that possess some of the characteristics associated with particular breast cancer subtypes, these cell lines are not ideal models as they do not reflect the pathophysiological process which are observed in human breast cancer tissue. A single cell line does not represent the cellular diversity and heterogeneity associated with breast tumours. Improvements could be made with single cell line models, by growing them in 3D cell culture as opposed to 2D cell culture, with the addition of specific matrices and perhaps with other cell types to closer represent breast

cancer. Although preliminary, our study could guide future directions upon which to focus using clinical samples to validate our *in vitro* associations.

Another limitations of this study was only performing optimisation studies once and not in triplicate, despite the fact that the doses selected were repeated more than three times in the critical experiments.

In summary, our data elaborate a mechanism to support the clinical studies suggesting the link between obesity and high cholesterol with an increased risk of breast cancer progression. LDL and 27OHC promote cell proliferation, migration and invasion through a novel mechanism in human breast cancer cells. This study shows that ERβ is a positive regulator of IGF-I and EGF pathways, that via their respective receptors can activate the MAPK/PI3K/AKT signalling pathways. The overall alterations in these pathways may contribute to increased proliferation rates and invasiveness of breast cancer cells and may identify novel opportunities for optimizing current breast cancer treatment regimens.

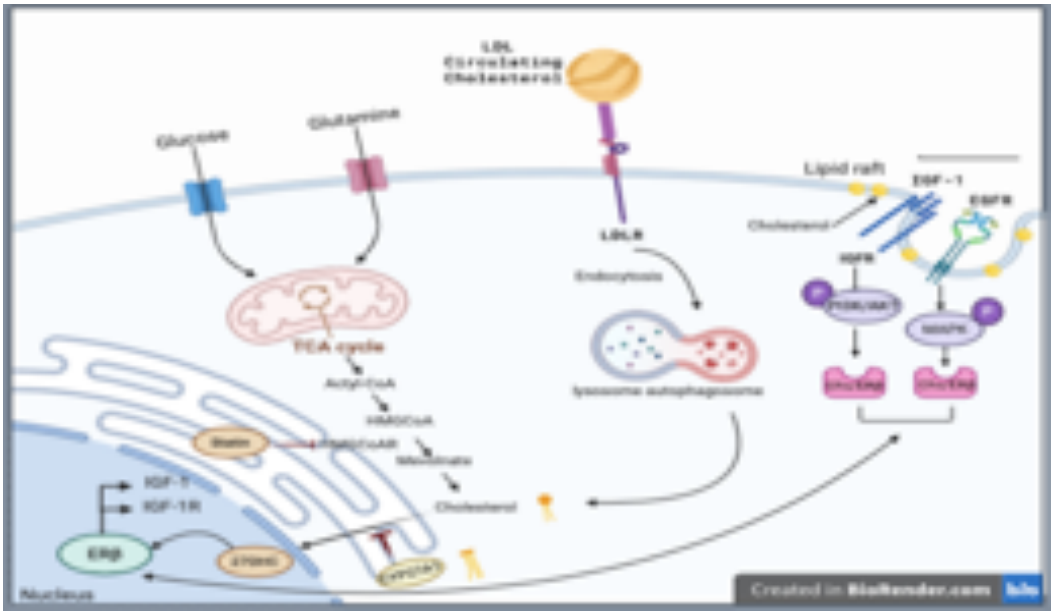


Figure 7. 1: Schematic illustrating the potential signalling pathways which underlie the phenotypic and metabolic changes that occur when breast cancer cells are exposed to high LDL:

Cholesterol metabolism in breast cancer is enhanced with LDL and 27OHC supporting breast cancer progression. The ERβ is central to these effects on invasion and migration and impacts on the IGF and EGF axes. This results in intrinsic activation of oncogenes such as IGF-I, which can bind to the IGF-IR and activate MAPK/ PI3K/AKT signalling pathways. The overall alterations in these pathways may contribute to increased proliferation rates and invasiveness of breast cancer cells.

7.2. Future direction

Work detailed in this thesis showed novel effects of LDL, which may contribute to increased growth, migration and invasion. In particular, LDL was demonstrated to regulate cross-talk between the ERβ and the IGF/EGF signalling pathway. Therefore, it will be important to further understand the underlying mechanistic role for ERβ in mediating the effects of LDL in TNBC, that use LDL to promote metastasis. This may identify new drug targets and optimise treatment regimens for this sub-set of TNBC patients.

- To assess whether ERβ expression correlates with disease free-survival and overall survival and if this affected by statin use and/or correlation with CAV-1, IGF-IR and EGF-R using tissue from TNBC patients.
- To determine whether the LDL-induced migratory and invasive phenotype of ERβ expressing TNBC cells is modulated in the presence of statins.
- To illustrate whether depleting cholesterol in cells causes a shift in raft and hormonal receptors and caveolar proteins to other parts of the plasma membrane. As ERα has previously been found to bind to CAV-1 (Schlegel *et al.*, 2001), ERβ may be present in the same signalling complex in TNBC.
- To investigate the role of other cholesterol metabolites on breast cancer progression such as 25-hydroxycholesterol.
- To understand how CYP27A1 impacts breast cancer progression as this may be a useful strategy to prevent or treat breast cancer, by using other types of experiments.

- To use Mendelian Randomization to determine whether CAV1, integrins and ERβ, which are associated with a TNBC metastatic phenotype, are causally associated with risk and development of TNBC breast cancer.

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